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PRINCIPAL INVESTIGATION: Frank J. Rauscher, Ph.D.

CONTRACTING ORGANIZATION: Wistar Institute
Philadelphia, Pennsylvania 19104-4268

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Frank J. Rauscher, Ph.D.

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Philadelphia, Pennsylvania 19104-4268

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We have identified a novel protein, BAP1, which binds to the BRCA1 RING finger domain (BRCA1-RF). BAP1 is a new, nuclear-localized, ubiquitin carboxyl-terminal hydrolase suggesting that deubiquitinating enzymes may play an important role in BRCA1 function. BAP1 binds to the wild-type BRCA1-RF both *in vitro* and *in vivo*, but not to mutant BRCA1-RF's found in tumors from breast cancer kindreds, or to related RING-finger proteins. BAP1 enhances BRCA1-mediated tumor suppression in colony formation assays and this activity requires the enzymatic function. BAP1 was mapped to human chromosome 3p21; rearrangements and intragenic mutations of BAP1 have been found in lung carcinomas, including a homozygous deletion. BAP1 is the first nuclear-localized ubiquitin carboxy-terminal hydrolase (of the UCH class) to be identified and may be a new tumor suppressor gene which functions in the BRCA1 growth control pathway.

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F. J. Rauscher III 1-17-91

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CHARACTERIZATION OF TWO PROTEINS WHICH INTERACT WITH THE BRACA1 GENE

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Introduction

The cloning of the chromosome 17q21 *BRCA1* breast cancer susceptibility gene is a landmark accomplishment in cancer genetics (Miki et al., 1994). Germline mutations in *BRCA1* appear to account for ~50% of familial breast cancers and essentially all families with 17q21-linked inherited susceptibility to ovarian and breast cancer (Szabo and King, 1995). The importance of this gene is underscored by the fact that kindreds segregating constitutive *BRCA1* mutations show a lifetime risk of 40-50% for ovarian cancer and >80% for breast cancer (Easton et al., 1993; Easton et al., 1995). The classification of *BRCA1* as a highly penetrant, autosomal dominant tumor suppressor gene has been genetically confirmed by the finding of frequent LOH of the wild-type allele in breast tumors from mutation carriers (Hall et al., 1990; Miki et al., 1994; Smith et al., 1992). Surprisingly, *BRCA1* mutations in sporadic breast cancer including those which show LOH have yet to be found and are extremely rare in sporadic ovarian cancer (Futreal et al., 1994; Merajver et al., 1995).

The *BRCA1* locus spans >100 kb comprising 24 exons (Miki et al., 1994). More than 100 constitutional mutations have been identified in *BRCA1* over the entire length of the gene and some clustering in populations and genotype-phenotype correlations have been suggested (FitzGerald et al., 1996; Ford et al., 1994; Muto et al., 1996; Roa et al., 1996, Struewing et al., 1995). The majority of germline mutations result in a truncated BRCA1 protein although recurrent missense mutations resulting in amino acid substitutions in kindreds have also been observed (Couch and Weber, 1996). The heterogeneity of BRCA1 mutant proteins produced by this spectrum of genetic mutations suggests that multiple, independent functions and/or protein-protein interaction surfaces are targets for mutational inactivation. However, the biochemical functions of BRCA1 are largely unknown.

The predominant BRCA1 mRNA of 8.0 kb encodes a 1863 amino acid protein with few sequence motifs suggestive of function (Miki et al., 1994). The most highly conserved regions are 1) the 100 amino acid N-terminus which encodes a RING finger motif which is predicted to bind zinc and may be a protein-protein interaction motif (Borden et al., 1995; Lovering et al., 1993); and 2) the C-terminus which contains an acidic region and a two copies of a novel motif, designated the BRCT domain, which is present in a variety of putative cell-cycle related proteins, including RAD9 and 53BP1 (Koonin et al., 1996). The most abundant BRCA1 protein is now accepted to be a ~220 kDa phosphoprotein which is predominantly, but apparently not exclusively, nuclear in subcellular distribution (Chen et al., 1995; Chen et al., 1996 Scully et al., 1996). Other isoforms of BRCA1 have been detected including a protein of 97 kDa which lacks exon 11, and thus a functional nuclear localization signal, and which is presumably the result of an alternative splicing event (Thakur et al., 1997). BRCA1 is localized to discrete nuclear dot structures in a cell-cycle dependent manner (Scully et al., 1997). These observations, coupled with the finding of a transcriptional activation domain in the BRCA1 C-terminus (Chapman and Verma, 1996) and the co-fractionation of BRCA1 with the RNA pol II holoenzyme (Scully et al., 1997) suggest a role in transcriptional regulation.

The expression patterns of BRCA1 further support its role in growth regulation and/or differentiation. The spatial-temporal expression pattern in the embryonic

mouse includes the neuroepithelium, and epithelial lineages of the skin, kidney and mammary gland (Marquis et al., 1995). Moreover, BRCA1 mRNA is sharply increased in alveolar and ductal cells of the breast epithelia during pregnancy (Marquis et al., 1995). Consistent with this, BRCA1 transcription is under (indirect) hormonal control in both cell culture and organismal systems (Gudas et al., 1995, 1996; Vaughn et al., 1996; Marks et al., 1997). BRCA1 is also highly expressed in the adult testis during the final stages of meiosis and spermiogenesis (Zabludoff et al., 1996). Together, these observations suggest a broad role for BRCA1 in terminal differentiation events in multiple tissues. Somewhat paradoxically, the murine *brca1 –/–* embryos die very early in gestation and exhibit severe cell proliferation defects and profound cell cycle arrest (Hakem et al., 1996 Liu et al., 1996). The association of BRCA1 expression with both proliferation and differentiation events suggests a possible role for BRCA1 in regulating a genetic program which prepares the cell for terminal differentiation and possibly maintains that phenotype thereafter. Results of cell culture and transfection studies have further underscored the tumor suppression function of BRCA1 without revealing much of that mechanism. BRCA1 antisense expression can transform fibroblasts and accelerates growth of breast cancer cell lines (Rao et al., 1996; Thompson et al., 1995). Expression of wild-type *BRCA1* inhibits colony function and tumor growth *in vivo*, whereas tumor derived mutations of *BRCA1* abolish this growth suppression (Holt et al., 1996).

Evidence of a role for BRCA1 as a terminal differentiation checkpoint has recently been provided by the finding that BRCA1 and the RAD51 protein (involved in DNA recombination/repair) are co-localized and physically associated in mitotic and meiotic cells (Scully et al., 1997). The co-localization of BRCA1 and RAD51 on synaptonemal meiotic chromosomes suggests a role for this complex in either the fidelity of DNA replication, cell cycle progression or genomic integrity. Though intriguing, these results do not suggest a function for BRCA1 which is lost in tumor-derived mutations in BRCA1. Strategies based upon identification of proteins which bind to BRCA1 have yielded components of the nuclear import pathway (Chen et al., 1996) and a novel RING finger/BRCT-domain-containing protein, BARD1 (Wu et al., 1996). However, none of these associated proteins have suggested a function for BRCA1.

We have chosen to focus upon the highly conserved BRCA1 RING finger domain as a potential protein-protein interface. This motif is defined by a spatially conserved set of cysteine–histidine residues of the form C_3HC_4. Structural analysis of the motif shows that two molecules of zinc are chelated by the consensus residues in a unique "cross-braced" fashion (for reviews, see; Klug and Schwabe, 1995; Saurin et al., 1996). Comparative structure analyses suggest that the RING fingers have a common hydrophobic core structure but that the region encoded by amino acids spanning cysteines 24 and 64 (for BRCA1) forms a highly variable loop structure which is the determinant of protein-protein interaction specificity. The RING motif occurs in over 80 proteins including the products of a number of proto-oncogenes and putative transcription factors (Saurin et al., 1996). Evidence that the RING finger domain functions as a protein-protein interface has come from the study of the proto-oncogene PML (Borden et al., 1995) and the transcriptional co-repressor KAP-1 (Friedman et al., 1996). Intriguingly, like BRCA1, both PML and KAP-1 are localized to discrete, non-overlapping, nuclear dot structures and mutations in the RING finger of PML abolish its localization to the nucleus (Borden et al., 1995).

We hypothesize that the BRCA1 RING finger is a binding site for protein(s) which either mediate BRCA1 tumor suppressor function or serve to regulate these functions. Genetic evidence supports this in that single amino-acid substitutions at metal chelating cysteines, C61G and C64G, occur in kindreds; these mutations segregate with the disease susceptibility phenotype and are predicted to abolish RING finger structure. We have used the yeast two-hybrid system to isolate proteins which directly bind to the wild-type BRCA1 RING finger but not to the C61G or C64G mutated RING fingers or other closely related RING fingers. We have isolated mouse and human clones of a novel protein, BRCA1 associated protein-1 (BAP1), which fulfills all of these criteria. BAP1 is a novel, nuclear localized, enzyme which displays the signature motifs and activities of a ubiquitin carboxy-terminal hydrolase. Full-length BRCA1 binds to BAP1 *in vitro* and *in vivo* and BAP1 enhances the growth suppression properties of BRCA1 in colony formation assays. The human *BAP1* locus was mapped to chromosome 3p21.3 and homozygous deletions of *BAP1* were found in non-small cell lung cancers. Together, these data suggest that *BAP1* is a key player in the *BRCA1* growth suppression pathway and may itself be a tumor suppressor gene. The identification of BAP1 as a ubiquitin hydrolase implicates the ubiquitin-proteasome pathway in either the regulation, or as a direct effector, of BRCA1 function. BAP1 is the first nuclear-localized ubiquitin carboxy-terminal hydrolase to be identified and may play a broad role in ubiquitin-dependent regulatory processes within the nucleus, including the emerging role of ubiquitin conjugation as a subcellular targeting signal.

BODY

I. Experimental Methods

Cell Culture, Transfections and Colony Formation Assays: COS1 and HEP2 cells were grown at 37°C, 5% CO_2 in DMEM supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. COS1 and HEP2 cells were transfected using DOSPOR transfection reagent (Boehringer Mannheim Biochemicals) following the manufacturers protocol. MCF7 cells were grown at 37°C, 5% CO_2 in DMEM supplemented with 10% FBS and non-essential amino acids.

Colony Formation Assay: MCF7 cells were transfected by a modified $CaPO_4$-DNA precipitation method (Holt et al., 1996). MCF7 cells, at $2X10^6$ cells/ 10 cm dish, were fed fresh medium approx. 3 hours prior to transfection and were then treated with the Ca-DNA precipitation. for 4 hours. The cells were subjected to a brief shock with transfection buffer containing 15% glycerol. Twelve to sixteen hours later, the cells were trypsinized, counted and plated directly into complete medium containing 0.75 mg/mL G418 at $5X10^5$ cells per 10 cm dish. Cells were fed fresh medium containing G418 every three to four days. Cells were stained for colonies approximately 21 to 28 days after transfection.

Yeast 2-hybrid: The yeast 2-hybrid system as modified by Stan Hollenberg was used for all yeast experiments (Vojtek et al., 1993). Two libraries were screened for interaction with LexA-BRCA1; a human B cell, oligo-dT-primed, cDNA library [(Durfee et al., 1993); a kind gift of Dr. Steve Elledge] and a mouse embryo (9.5-10.5 day), random-primed, cDNA library size selected for inserts of 300 to 500 base pairs in length [(Vojtek et al., 1993); a kind gift of Dr. Stan Hollenberg].

Construction of expression plasmids:

LexA fusion constructs: The first 100 amino acids of human BRCA1 (BRCA1-RF) was used as the "bait" to screen for interacting proteins. The BRCA1-RF domain was made from overlapping oligonucleotides whose sequence had been optimized for expression in *E. coli* and *S. cerevesae* (Madden, 1991 #44). Double-stranded DNA was generated by the polymerase chain reaction (PCR) and amplified with "outside" primers containing EcoRI and SalI enzymatic restriction sites (BRCA1-RF-5' oligonucleotide 5'-GCTAGAATTCACCATGGACCTGTCTGCTCTG-3'; BRCA1-RF-3' oligonucleotide 5'-GCTAGTCGACTTCCAGACCAGTGTCCAG-3'). A "wild-type" BRCA1-RF domain was confirmed by sequencing. The BRCA1-RF domain point mutants, BRCA1-C64G (Cys 64 to Gly) and BRCA1-C61G (Cys 61 to Gly), were created by PCR-mutagenesis using the "outside" primers described above and overlapping oligonucleotides containing the appropriate nucleotide change (BRCA1-C61G-sense 5'-CCATCTCAAGGTCCACTGTGTAAG-3'; BRCA1-C61G-antisense 5'-CTTACACAGTGGACCTTGAGATGG-3'; BRCA1-C64G-sense 5'-CAATGTCCAC TGGGTAAGAACG ACATC-3'; BRCA1-C64G-antisense 5'-GATGTCGTTCTT ACCCAGTGGACATTG-3'; Ho et al., 1989). The BRCA1-delAG185 mutant was generated by PCR using the BRCA1-RF-5' oligonucleotide and a 3' oligonucleotide that encoded the changed amino acid sequence (5'-GCATGGATCCTCAAACCTTGT GCAGGCAGGTACCCTGGTCAACAGGAGACAGGTGGGAAACCAGGATCTTTTGCATA GC-3'). The BRCA1-del31 truncation mutant was a mis-primed PCR reaction of

BRCA1-RF identified by sequencing. All LexA fusion constructs (wild-type and mutant) were made by cloning the appropriate BRCA1-RF domain into the vector pBTM-116 (Vojtek et al., 1993). The LexA-RPT-1 construct (amino acids 1-100) was made by PCR-mediated amplification of the corresponding nucleotides of a RPT-1 PCR sample [(Patarca et al., 1988); kindly provided by Dr. Harvey Cantor] followed by enzymatic digestion and ligation into the pBTM-116 vector. All clones were confirmed by sequencing. Expression of all constructs in yeast was confirmed by Western analysis using antibodies against the LexA DNA-binding domain (data not shown).

BAP1 constructs: A partial BAP1 cDNA (EST-BAP1) is encoded by two overlapping EST clones which were obtained from the Image Consortium [I.M.A.G.E. Consortium (LLNL) cDNA Clones #46154 and #40642; (Lennon et al., 1996)]. This partial BAP1 clone was generated by digesting clone #40642 with HindIII and FspI and clone #46154 with FspI and EcoRI. These two pieces were then ligated into the HindIII and EcoRI sites of the vector pcDNA3 (Invitrogen). Reverse-transcriptase-PCR using a gene-specific primer (5'-GAAGCGGATGTCGTGGTAGG-3') was used to identify 62 nucleotides which were missing from the "EST-BAP1" cDNA. These 62 nucleotides were inserted into the "EST-BAP1" cDNA by digestion of the RT-PCR product with the restriction enzymes KpnI (a unique site within the 5' RT-PCR oligonucleotide; 5'-CCTGTTATTAACCCTCACTAAAGGGAAGGGTACCATGAA TAAGGGCTGGCTGGAGC-3'; 3' RT-PCR-oligonucleotide 5'-GAAGCGGATGTCGTGGT AGG-3') and AvrII followed by ligation with AvrII+EcoRI digested "EST-BAP1" cDNA into KpnI-EcoRI digested pcDNA3. GST-hBAP1(483-729) was generated by cloning nucleotides 1486 to 3525 (the original two-hybrid clone) into pGEX-5x-1 (Pharmacia Biotech, Inc.). GST-hBAP1(438-594) and pACT-hBAP1(438-594) {nucleotides 1486-1821} were generated and amplified by PCR (pACT 5'-vector primer 5'-GATGTATATAACTATCTATTCG-3'; BAP1-*trunc.* oligonucleotide 5'-GCATAGATCTT CACCCCTGGCTGCCTTGGATTGG-3'), digested with restriction enzymes and ligated into the appropriate vector.

Mapping of BRCA1/BAP1 interaction domain: Truncations of mBAP1(596-721) were performed by PCR-based mutagenesis. The appropriate region of mBAP1(596-721) was amplified by PCR using a vector primer (pVP16 5'-primer, 5'-CCGATGCCCTT GGAATTGACGAG-3'; pVP16 3'-primer, 5'-CGATGAATTCGAGCTAGCTTCTATC-3') and the appropriate truncating oligonucleotide (Mc43Ct1, 5'-GCATGAATTCTCAGCT CCGGCGCACTGAGATG-3'; Mc43Ct2, 5'-GCATGAATTCTCAAGCCAGCATGGA TATGAAGG-3'; Mc43Ct3, 5'-GCATGAATTCTCAGTCATCAATCTTGAACTTC-3'; Mc43Ct4, 5'-GCATGAATTCTCATGCAATCTCGGCTTCTAC-3'; Mc43Nt1, 5'-GCATG GATCCCCAAGATTGATGACCAGCGAAGG-3'). The product was then ligated into the mouse library-yeast expression vector, pVP16. The point mutant mBAP1(L691P) was made by standard PCR-based mutagenesis protocols (Mc43(L691P) sense-primer, 5'-GCTGGCCAACCCGGTGGAACAG-3'; Mc43(L691P) antisense-primer, 5'-CTGTT CCACCGGGTTGGCCAGC-3'; Ho et al., 1989) using the same vector primers described above. All clones were confirmed by sequencing and expression in yeast was confirmed by Western analysis using antibodies against the VP16 activation domain (data not shown).

Tissue Northerns: Tissue RNA blots were obtained from Clonetech Laboratories, Inc. (Palo Alto, CA). Blots were hybridized with ^{32}P-labeled hBAP1(483-729) cDNA (nucleotides 1486 to 3525) using standard protocols.

Fluorescent *In Situ* Hybridization (FISH): FISH using a biotin-labeled 3.5 kb cDNA (full-length) clone of BAP1, with corresponding DAPI-banding and measurement of the relative distance from the short arm telomere to the signals (FLpter value) was performed as described previously (Tommerup and Vissing, 1995).

Immunolocalization: All immunofluorescence was performed as previously described (Ishov and Maul, 1996). BAP1 polyclonal antibodies were detected with FITC using biotin-avidin enhancement. Cells were stained for DNA with bis-benzimide (Hoechst 33258, Sigma Chemical Co.) and mounted using Fluoromount G (Fisher Scientific). Analysis was performed with a confocal scanning microscope (Leica, Inc.).

BAP1 Protein Characterization:

Generation of antibodies: Using PCR cloning (pACT 5'-vector primer 5'-GATGTATATAACTATCTATTCG-3'; BAP1(antibody) 5-CGTAGTCGAC TGTCAGCGCCAGGGGACTC-3'), the cDNA region encoding amino acids 483 to 576 of BAP1 was fused downstream of the 6 Histidine residues of the vector pQE-30 (QIAGEN Inc.). The His-tagged protein was purified from *E. coli* over a Ni-agarose column as previously described (Friedman, 1996 #76) and was used to immunize rabbits for the production of polyclonal antibodies (Cocalico Biologicals, Inc.). Immunoprecipitation of BAP1 was performed by previously described procedures for the metabolic labeling and immunoprecipitation of proteins from cell lysates (Friedman, 1996 #76).

In Vitro Protein Association: GST, GST-hBAP1(483-729) and GST-hBAP1(483-594) were expressed in *E. coli* and then purified as described (Frangioni and Neel, 1993). The ^{35}S-LexA-BRCA1-RF and ^{35}S-BRCA1 were produced *in vitro* via coupled transcription/translation (TNT®, Promega Corp.). Association between proteins was assayed as described by Barlev et al. (1995).

BAP1 Enzymatic Assay: Assays for BAP1 enzymatic activity were performed essentially as described for the UCH-L1 and UCH-L3 enzymes (Mayer and Wilkinson, 1989). Briefly, bacteria (DH5a) harboring an IPTG-inducible expression plasmid containing BAP1 (pQE-30; QIAGEN Inc.) were grown and induced with 1 mM IPTG for 4 hours. The bacteria were collected and the pellets were resuspended to 1/20 volume (original culture) in lysate buffer (50 mM Tris, pH 8.0, 25 mM EDTA, 10 mM 2-mercapto-ethanol, 100 μg/ml lysozyme). The lysates were sonicated and centrifuged at 40,000 Xg. The soluble fractions were used for subsequent activity assays. The pellets were resuspended in an volume equal to that of the supernatant and samples of both pellet and supernatant were analyzed by SDS-PAGE for expression levels and inclusion body formation.

Assays for ubiquitin carboxy-terminal hydrolase activity were performed using the glycine 76 ethyl ester of ubiquitin (Ub-OEt) as a substrate (Mayer and Wilkinson, 1989; Wilkinson et al., 1986). Assay reactions (20 μL) contained 3.0 μg Ub-OEt and sample enzyme in assay buffer (50 mM Tris pH 7.6, 100 μg/ml Ovalbumin, 10 mM DTT), and

were incubated at 37°C for 10 min. Reactions were stopped by addition of 80 µl of the HPLC solvent followed by refrigeration. Ester hydrolysis was detected by C8 reverse phase HPLC with isocratic 45% acetonitrile in 50 mM perchlorate (pH 2) as solvent. Assays were done in triplicate. The peak areas were integrated and normalized with respect to a ubiquitin standard.

Mutation Screening:

RNA/DNA Preparation: Genomic DNA was prepared from breast and lung cancer cell lines using standard methods. Total RNA was extracted by the cesium chloride-ultracentrifugation method (Ausubel et al., 1987). First strand cDNAs were synthesized from RNA by M-MLV reverse transcriptase (Gibco BRL) according to the manufacturer's instructions.

Southern and Northern Blot Hybridization: Five µg of genomic DNA, subjected to restriction enzyme digestion, or ten µg total RNA, was electrophoretically gel-fractionated and transferred to Hybond N+ membranes (Amersham). Hybridization was performed with a radiolabeled full-length BAP1 cDNA probe followed by washes under standard conditions and detection by autoradiography.

Single Strand Conformational Polymorphism (SSCP) Analysis: Seventeen overlapping PCR primer pairs, each with a predicted product size of approximately 200 base pairs, were designed to span the 2.2 kb open reading frame of the BAP1 cDNA sequence. One µl cDNA (from RNA) was then amplified in 20 µl PCR reactions containing 20 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl, 0.2 mM each dNTP, 0.1 mM each forward and reverse primer, 0.05 ml ^{32}P-α dCTP, and 0.5 units Taq DNA Polymerase (BRL). PCR reactions were carried out in a Perkin-Elmer 9600 Thermocycler using a touchdown technique: a 2.5 min. initial denaturation at 94°C was followed by 35 cycles of denaturation at 94°C x 30s, annealing, initially at 65°C decreasing by 1°C for each of the first ten cycles to 55°C, x 30s, and extension at 72°C x 30s with a final extension of 5 min at 72°C. PCR products were then diluted 1:10 with SSCP dye (95% formamide, 20 mM EDTA, and 0.05% each of bromophenol blue and xylene cyanol), heat-denatured, and electrophoresed on 0.5X MDE gels +/- 10% glycerol. Abnormal single-stranded DNA detected as autoradiographic shifts were reamplified by PCR and subjected to automated dye-terminator sequencing (ABI 373).

II. Results

A Yeast Two-Hybrid Screen for BRCA1 RING Finger Interacting Proteins

The mouse and human BRCA1 RING finger regions are shown in Fig. 1A and are compared to related RING domains. The signature C_3HC_4 structure is highlighted. The RING finger from the RPT-1, a putative lymphocyte-specific transcription factor, is most highly related to BRCA1 (Patarca et al., 1988). We constructed a totally synthetic BRCA1 gene encoding the amino-terminal 100 amino acids of human BRCA1 using long oligonucleotides and PCR-mediated overlap-extension gene synthesis techniques (Madden, 1991 #44). Codon usage was optimized for expression in *E. coli* and *S. cerevisiae*. The resulting gene was fused to the LexA DNA binding domain (Fig. 1B). The negative control/specificity controls included 1) the Cys61Gly and Cys64Gly substitutions of BRCA1 which occur in breast cancer pedigrees, 2) the protein equivalent of the del AG185 mutation which results in a frame shift at amino

acid 22 followed by 17 out-of-frame amino acids and a stop codon, 3) a truncated BRCA1 RING finger at amino acid 31, the result of a PCR error, 4) the RPT-1 RING finger domain, and 5) a non-specific control LexA fusion with RhoB. The wild-type BRCA1 RING finger (BRCA1-RF) did not display intrinsic transcriptional activation function in yeast and proper expression of each LexA fusion in yeast was confirmed by Western blot analysis (data not shown).

Guided by the expression patterns of BRCA1 during mouse development and in human spleen, we chose to screen cDNA libraries constructed from E9.5-10.5d whole mouse embryos and human adult B cells with the LexA-BRCA1-RF. Thirty-one cDNAs which specifically interacted with BRCA1-RF were obtained: 8 of these (3 from the human library and 5 from the mouse library) encoded the same amino acid sequence and were further pursued. Each clone shares the same translational reading frame with the transcriptional activation domain to which it is fused and in addition, the fusion junctions were different among the clones suggesting that the interaction was not due to a fusion-junction artifact. Fig. 2A shows a representative secondary screen for a subset of these clones performed by re-introducing the indicated purified plasmids into naive yeast. Each clone showed a strong interaction with the BRCA1-RF but failed to interact with the C64G, C61G (data not shown), del31, delAG (data not shown), RPT-1, RhoB, or any of the specificity control LexA fusions (data not shown). The longest cDNA retrieved in the two-hybrid screen was a ~2.0 kb clone from the human library and encoded 246 amino acids followed by a 1.3 kb 3'UTR. Each mouse clone encoded an overlapping smaller subset of this human open reading frame which served to partially map the minimal interaction domain. A more detailed analysis of the BRCA1/BAP1 interaction is discussed below and shown in Fig. 2B. Interestingly, the mBAP(518del718) clone interacted most poorly with BRCA1-RF and lacked a 93 bp sequence (the reading frame was maintained), possibly the result of a naturally occurring splice variant. That these clones fail to bind multiple, independent tumor-derived mutations of the BRCA1-RF provides strong genetic evidence for their relevance to the functions of BRCA1.

Analysis of the BAP1 cDNA

We designated this gene product BRCA1 associated protein (BAP1): A nearly full-length cDNA was constructed via a combination of cDNA library screening, EST database searching, 5'RACE and RT-PCR (Fig. 3A). The current BAP1 cDNA comprises 3525 bp; a polyA tract is present along with multiple polyA signals. Conceptual translation yields a long open reading frame of 729 amino acids with a predicted MW of 81 kDa and pI of 6.3. The presumptive initiator methionine is within a favorable context for translation start, however the short 5'UTR of 39 bp encodes amino acids in-frame with the presumptive methionine and does not contain a stop codon. BLAST searches indicated that BAP1 is a novel protein with motifs suggestive of function. The amino-terminal 240 amino acids show significant homology to a class of thiol proteases, designated ubiquitin C-terminal hydrolase (UCH), which are implicated in the proteolytic processing of ubiquitin (Wilkinson et al., 1989). These enzymes play a key role in protein degradation via the ubiquitin-dependent proteasome pathway. The most closely related UCH is a hypothesized protein from *C. elegans* which shares 63% similarity (40% identity) with BAP1 through the UCH domain and is also likely to be a UCH enzyme (Figs. 3B and 3C). Pairwise similarities to other mammalian UCHs of 54% (UCHL3) and 56% (UCHL1) have also been found

(Figs. 3B and 3C). Most importantly, the residues which form the catalytic site (Q85, C91, H169, and D184) are completely conserved, including the FELDG motif (Larsen et al., 1996). In addition, a loop of highly variable sequence, which is disordered in the crystallographic structure of human UCH-L3 (Johnston et al., 1997), is present (residues 140 to 167). This loop may occlude the active site or provide substrate specificity for the enzyme.

BAP1 has a number of additional motifs; a region of extreme acidity spanning amino acids 396 to 408, as well as multiple potential phosphorylation sites and N-linked glycosylation sites (Figs. 3A and 3B; S. Subbiah, personal communication). The C-terminal one-third is highly charged and is rich in proline, serine and threonine. The extreme C-terminus contains two putative nuclear localization signals, KRKKFK and RRKRSR, and is hydrophilic; it is predicted to fold into a helical (possibly coiled-coil) structure (Figs. 3A and 3B). Indeed, definition of the BAP1-BRCA1 interaction by deletion mutagenesis supports this hypothesis. Deletion of protein sequence from the carboxy or amino termini of mBAP1(596-721) (the "minimal interaction domain") almost completely destroyed the BAP1-BRCA1 interaction (Fig. 2B). Furthermore, within the BAP1 minimal interaction domain, the mutation of leucine 691 to a proline, predicted to disrupt the helical nature of this region, disrupted the BAP1-BRCA1 interaction, consistent with the hypothesis that BAP1 uses a coiled-coil domain to interact with the RING finger domain of BRCA1. This overall architecture suggests that BAP1 may be a novel, nuclear-localized, member of the UCH enzyme family.

BAP1 is a 90 kDa Protein With UCH Activity

The BAP1 open reading frame encodes a protein of 81 kDa predicted molecular weight. Expression of the cDNA *in vitro* and analysis by SDS-PAGE yielded a single major protein with an apparent molecular weight of 90 kDa (Fig. 4A). Immunoprecipitation of this product with anti-BAP1 antiserum confirmed that the protein expressed *in vitro* resulted in a polypeptide that contained the antigen used to raise the antibody. Furthermore, expression of the BAP1 cDNA in COS1 cells followed by immunoprecipitation of ^{35}S-labeled whole cell extracts also yielded a protein with an apparent molecular weight of 90 kDa (Fig. 4A). The difference between apparent and predicted molecular weights may be accounted for by the unusual properties of the C-terminus or by post-translational modifications; however, this has yet to be determined.

To determine whether BAP1 did indeed have UCH activity, the BAP1 cDNA was expressed in bacteria and this protein was assayed for the ability to hydrolyze the glycine 76 ethyl ester of ubiquitin (Ub-OEt; Mayer and Wilkinson, 1989). Overexpression of BAP1 in bacteria led to abundant protein, most of which was found in an inactive, insoluble form (Fig. 4B, lower panel, compare uninduced and induced precipitates). The BAP1 protein found in the soluble fraction was able to hydrolyze Ub-OEt and the level of this activity increased with the level of protein (Fig. 4B, lower panel) indicating that BAP1 contains UCH-like enzymatic activity. The active site thiol residue responsible for UCH activity in UCH-L3 has been identified and its mutation leads to abolition of enzyme activity (Larsen et al., 1996). Mutation of the corresponding cysteine residue in BAP1, BAP1(C91S), yielded a protein with no UCH activity (Fig. 4C) further suggesting that BAP1 is a thiol protease of the UCH family.

BAP1 Associates With BRCA1 *in vitro*

Association of the BRCA1-RF with BAP1 was confirmed *in vitro* by binding of ^{35}S-labeled LexA-BRCA1-RF to hBAP1(483-729) fused to glutathione S-transferase (GST; Fig. 5A). The labeled BRCA1-RF specifically bound to the GST-hBAP1(483-729) fusion protein, but not to GST alone (Fig. 5C) confirming a physical association of the two proteins. To confirm that the association of the BRCA1-RF to BAP1 was not an artifact of using only a portion of BRCA1, full-length BRCA1 was expressed *in vitro* and incubated with GST and GST-hBAP1(483-729) (Fig. 5B). As a further control for the specificity of the interaction, BRCA1 was also incubated with GST-hBAP1(483-594), a GST-BAP1 fusion protein lacking the minimal interaction domain (see Figs. 2A and 3). The BRCA1 protein specifically bound to GST-hBAP1(483-729) and not to GST or GST-hBAP1(483-594) (Fig. 5B), confirming the direct interaction of BRCA1 with BAP1 through the C-terminal region of BAP1.

BAP1 is a Nuclear Protein Expressed in a Variety of Tissues

A direct interaction between BAP1 and BRCA1 suggests that BAP1 might be expressed in an overlapping subset of tissues expressing BRCA1 and that the subcellular location of BAP1 and BRCA1 may be the same. The expression of BAP1 in a variety of human adult tissues was determined by Northern blot analysis. Probing these blots with a 2.0 kbp radiolabeled partial BAP1 cDNA (nucleotides 1488 to 3525) indicated that the mRNA encoding BAP1 was present as a single mRNA species of ~4 kb in all tissues except testis, where a second, ~4.8 kb mRNA, was also detected (Fig. 6A). Highest expression was detected in testis, placenta and pancreas with varying levels detected in the remaining tissues. Expression of BAP1 in normal breast tissue was confirmed by RT-PCR of total RNA isolated from HUMEC cells (data not shown). The level and pattern of tissue expression shown by BAP1 is similar to that shown by BRCA1 (Miki et al., 1994).

The location of BAP1 within the cell was determined by immunofluorescence microscopy. HEP2 epithelial cells were transfected with the BAP1 cDNA and analyzed by immunofluorescence staining with αBAP1 antibodies. BAP1 was found almost exclusively in the nucleus of the cell (Fig. 6B; green staining of the two leftmost cells), consistent with 1) its association with BRCA1, and 2) the presence of 2 nuclear localization signals in the BAP1 protein sequence.

BAP1 Augments the Growth Suppressive Activity of BRCA1

The biochemical function of BRCA1 is currently unknown, however, several studies have shown that BRCA1 can affect the growth characteristics of cells (Holt et al., 1996; Rao et al., 1996). To determine whether BAP1 may affect cell growth itself or may affect BRCA1-mediated changes in cell growth, BRCA1 and BAP1 cDNAs were co-transfected into MCF7 breast cancer cells (Fig. 7). This cell line was chosen for several reasons; one, it has been previously shown that these cells are inhibited by the overexpression of BRCA1 (Holt et al., 1996); two, both northern and RT/PCR analyses showed that BAP1 was expressed in this cell line (data not shown); and three, analysis of the open reading frame from BAP1 cDNA prepared from this cell line showed no mutations (data not shown).

The expression of BRCA1 alone (BRCA1:pCMV5) decreased the number of colonies formed by these cells when compared to the vector control (pcDNA3:pCMV5), in agreement with other studies (Holt et al., 1996). The co-expression of BRCA1 and BAP1 (BRCA1:BAP1) significantly decreased the number of cell colonies (approximately 4-fold vs. BRCA1 alone; see Fig. 7B) indicating that BAP1 enhances the growth suppressive actions of BRCA1. A mutant of BAP1, BAP1(165-729), in which the enzymatic region is deleted but which still binds to BRCA1 (data not shown), also enhanced the growth suppression of BRCA1, but not to the same extent as the wild-type BAP1.

In contrast to BRCA1, the expression of BRCA1-D11 (BRCA1 missing the 11th exon) in MCF7 cells by itself had no effect on the growth of MCF7 cells (Fig. 7). However, the co-expression of BRCA1-D11 and BAP1 significantly decreased the number of colonies, suggesting that the presence of BAP1 could functionally substitute for the missing 11th exon of BRCA1 and/or that BAP1 itself was an inhibitor of cell growth. In support of this latter hypothesis, the expression of BAP1 in MCF7 cells did somewhat reduce the number of colonies formed (pcDNA3:BAP1; see Fig. 7B). The expression of the enzymatic mutant, BAP1(165-729), alone or in combination with BRCA1-D11 yielded the same number of colonies. Thus, enzymatically active BAP1 enhances BRCA1-mediated suppression of growth.

BAP1 is Located on Chromosome 3p21.3 and is Mutated in Non-Small Cell Lung Carcinoma.

The possibility that BAP1 may be a tumor suppressor gene suggested that its deletion might play a critical role in tumor pathogenesis. We questioned whether BAP1 might be located at a chromosomal region routinely mutated in breast cancer. The full-length BAP1 cDNA was used in fluorescent *in situ* hybridization (FISH) to identify the chromosomal location of the *BAP1* gene (Fig. 8). Specific signals were observed only on the midportion of chromosome 3 with 42 of 69 analyzed metaphase spreads showing at least one specific signal. The FLpter value was 0.27 ± 0.02, corresponding to a localization for BAP1 at 3p21.1-p21.31. This location is a region of LOH for breast cancer as well as a region routinely deleted in lung carcinomas (Buchhagen et al., 1994; Thiberville et al., 1995).

The chromosomal location of *BAP1* suggested the possibility of mutations within *BAP1* in tumor samples. Thus, a variety of tumor samples were screened for mutations within the *BAP1* gene by Southern, Northern and PCR-based SSCP analyses. Genomic DNAs from a panel of small cell lung cancer (SCLC), non-small cell lung cancer (NSCLC), breast cancer, and lymphoblastoid cell lines, were subjected to EcoR1 digestion and then hybridized to a radio-labeled, full-length BAP1 probe. A single 23 kb band was detected in the lymphoblastoid and most tumor cell lines (data not shown). One NSCLC line, H226, did not show the 23 kb band but did show an aberrant approximately 30 kb band (data not shown; This finding was confirmed in a second experiment using freshly prepared genomic DNA from this cell line). To further characterize this potential genomic rearrangement, we subjected genomic DNA from H226 and a smaller number of lung cancer and lymphoblastoid lines to BamH1 digestion. Using the full-length BAP1 probe, we detected four distinct bands at 7.5 kb, 4.0 kb, 3.0 kb, and 2.4 kb which were present in all cell lines tested with the exception

of H226 (Fig. 9A). In the H226 line, we detected only the 2.4 kb band and an aberrant 2.6 kb band.

Further mutational analysis of BAP1 was performed by screening a panel of lung cancer and lymphoblastoid cell lines for expression of the BAP1 mRNA. Northern analysis showed that most cell lines expressed a single ~4 kb mRNA (Fig. 9B). However, two cell lines, H226 and H1466 (both NSCLCs), showed undetectable levels of BAP1 expression (a not unexpected result for sample H226) suggesting that BAP1 may play a critical role in NSCLC pathogenesis.

Finally, a panel of lung and breast cancer cell lines were screened for mutations in the BAP1 open reading frame by RTPCR-SSCP (Fig. 9C). We detected a homozygous 8 base pair deletion in the cDNA from the NSCLC line H1466. This short deletion leads to a frameshift and encodes a 393 amino acid protein. This homozygous deletion was confirmed to be present in genomic DNA from the same cell line. These data clearly show that genetic alterations, including intragenic homozygous deletions, occur in BAP1.

III. Discussion:

We have discovered and characterized a novel protein, BAP1, which binds to the BRCA1 RING finger motif. A number of lines of evidence are offered which support a role for BAP1 in BRCA1 signal transduction pathways. Specifically, we showed that: 1) BAP1 binds to the RING finger of BRCA1, but not to tumor-derived mutants of BRCA1 or related RING domains; 2) The BAP1-BRCA1 interaction occurs *in vitro* and BAP1 mRNA is expressed in those tissues which also express BRCA1; 3) BAP1 is a nuclear-localized, ubiquitin carboxy-terminal hydrolase (UCH) which cleaves model ubiquitin substrates *in vitro*; 4) BAP1 enhances BRCA1-mediated suppression of cell growth in colony formation assays and does so in a manner dependent upon the UCH enzymatic domain and the BRCA1-interaction domain; and 5) Human BAP1 maps to 3p21.3 and intragenic, homozygous deletions occur in non-small cell lung cancer cell lines. Together, these observations suggest that BAP1 is a tumor suppressor gene and that it serves either as a regulator or an effector in BRCA1 growth control pathways. Both the specificity of the BRCA1 RING finger-BAP1 interaction and the fact that independent, tumor-derived missense mutations in the cysteines in the BRCA1 RING finger domain abolish interaction with BAP1 provide compelling evidence for the physiological relevance of this interaction.

Any discussion of mechanisms of BRCA1-BAP1 mediated growth control must center on the fact that BAP1 is a protease of the ubiquitin carboxy-terminal hydrolase (UCH) family. This discovery immediately implies a role for either ubiquitin-mediated, proteasome-dependent degradation or other ubiquitin-mediated regulatory (Isaksson et al., 1996) pathways in BRCA1 function. Regulated ubiquitination of proteins and subsequent proteasome-dependent proteolysis plays a role in almost every cellular growth, differentiation and homeostatic process (reviewed by Ciechanover, 1994; Isaksson et al., 1996; Wilkinson, 1995). This pathway can be broadly subdivided into reactions involving 1) pro-ubiquitin processing and ATP-dependent activation of ubiquitin; 2) substrate recognition, conjugation and editing of the polyubiquitin chain; 3) proteasome-dependent degradation of the ubiquitin protein and; 4) cleavage/debranching of peptide-ubiquitin conjugates and recycling of ubiquitin to

cellular pools. The pathway is regulated at almost every step. First, at the level of substrate specificity via the concerted actions of activating enzymes, carrier proteins and ligation enzymes, and secondly, at the level of proteolytic deubiquitination and ubiquitin hydrolysis. The latter enzymes are ubiquitin-specific thiol proteases which can be broadly classified into two families: the ubiquitin-specific proteases (UBPs) and the ubiquitin carboxy-terminal hydrolases (UCHs).

The UBP family members are 50-300 kDa, cytoplasmic or nuclear-localized proteins which, in general, cleave ubiquitin or ubiquitin-conjugates from large substrates. Such enzymatic activity can be found directly associated with the 26S proteasome and may serve a regulatory function by editing ubiquitin on large substrates or cleaving polyubiquitin, thus replenishing ubiquitin pools (Lam et al., 1997). Remarkably, a number of UBPs have been isolated as growth regulatory and/or developmental control genes such as DOA4 in yeast, which controls DNA replication and repair (Papa and Hochstrasser, 1993); UBP3 which is involved in transcriptional silencing in yeast (Moazed and Johnson, 1996); the TRE2 oncogene which is mutated in the UBP active site and functions as a dominant negative transforming gene (Nakamura et al., 1992); the drosophila *Fat Facets* gene which controls pattern formation and eye development (Huang et al., 1995; Huang and Fischer-Vize, 1996); and the human DUB family of cytokine-inducible UBPs which control hematopoietic differentiation (Zhu et al., 1996, 1997).

By contrast, the UCH family has been characterized as a set of small (25-30 kDa) cytoplasmic proteins which prefer to cleave ubiquitin from ubiquitin-conjugated small substrates and may also be involved in the co-translational processing of proubiquitin. Like the UBPs, UCHs show considerable tissue specificity and developmentally-timed regulation (Wilkinson et al., 1992). UCH family members are strongly and differentially expressed in neuronal, hematopoietic and germ cells in many species. Most remarkably, a novel UCH enzyme has recently been cloned from *Aplysia Californicus* whose enzymatic function is essential for acquisition and maintenance of long-term memory (Hedge et al., 1997). Finally, UCH levels are strongly down-regulated during viral transformation of fibroblasts (Honore et al., 1991), consistent with a role in growth control.

BAP1 is the newest member of the UCH family and considerably expands the potential roles of this family of proteases. BAP1 is a much larger protein (90 kDa) and is the first nuclear-localized UCH. In addition to containing the ~250 amino acid amino-terminal UCH catalytic domain, it includes a long carboxy-terminal extension with rich in proline, serine and threonine and a short, highly acidic region, elements which may confer a short half-life (Rechsteiner et al., 1996). The extreme carboxy-terminus encodes two potential nuclear localization signals which overlap the approximately 125 amino acid BRCA1 interaction domain. It was this domain that was independently isolated from mouse and human libraries in the two-hybrid screen and is predicted to fold into a long amphipathic helix of coiled-coil character, the structure of which may be important for BRCA1 interaction: Substitution of proline 691 with a leucine abolishes the BAP1-BRCA1 interaction. We have also detected a potential splice variant in BAP1 which results in loss of 31 amino acids of the BRCA1 interaction domain and greatly reduces the ability of BAP1 to bind the BRCA1 RING finger, further suggesting that the BAP1-BRCA1 interaction is regulated. Thus, our data suggest that

the BAP1 carboxy-terminus is tethered to BRCA1 via the RING finger domain and that the UCH catalytic domain is free to interact with ubiquitin substrates.

A simple model explaining most of our data is that BRCA1 is a direct substrate for the UCH activity of BAP1. Thus, in contrast to all of the known UCHs which are comprised entirely of the UCH domain, the carboxy-terminal extension provides substrate and/or targeting specificity for the catalytic function. Paradigms for separate substrate recognition and catalytic domains occur throughout the ubiquitin conjugation/ligation system (see Wilkinson, 1995 and references therein). Regulated ubiquitination of BRCA1 and subsequent proteasome-mediated degradation would not be surprising given that both BRCA1 levels and subnuclear localization are tightly regulated in the mitotic cell-cycle and during meiosis (Gudas et al., 1996; Scully et al., 1997; Zabludoff et al., 1996). BAP1-mediated deubiquitination of BRCA1 would be expected to stabilize the protein and protect it from proteasome-mediated degradation. This scenario is consistent with both the ability of co-transfected BAP1 to enhance the tumor suppressor effects of BRCA1 in colony formation assays and the finding of mutations in BAP1 in cancer cell lines.

A second, and equally plausible, hypothesis is that the BRCA1-BAP1 association serves to target the UCH domain to other substrates. These substrates may be bound to other sites on BRCA1; in this scenario, BRCA1 could be construed as an assembly or scaffold molecule for regulated assembly of multiprotein complexes, a function which has been postulated for other tumor suppressor proteins (e.g. pRb; Sellers and Kaelin, 1996; Welch and Wang, 1995). BAP1 could thus be a regulator of this assembly via controlled ubiquitin proteolysis. In this context, it is interesting to note that two other RING finger-containing proteins are involved in controlled proteolysis processes which depend upon the integrity of the RING finger structure: 1) the mouse homologue of the drosophila *seven-in-absentia* (a RING finger protein) binds to the tumor suppressor protein Deleted in Colon Cancer (DCC), thus targeting it for proteasome-mediated degradation. This degradation requires the RING finger structure (E. Fearon, personal communication). 2) The herpes virus protein VMW110 RING finger protein binds directly to a UBP family member, HAUSP, and appears to target it to the ND10/POD nuclear dot structure, which itself contains the RING finger-containing proto-oncogene PML (Everett et al., 1997).

In this context, it is interesting to note that BRCA1 is also localized in nuclear dot structures in a cell-cycle dependent manner (Scully et al., 1997) This association of BRCA1 with RAD51 in both mitotic and meiotic cells broadly implicates BRCA1 in DNA repair and/or recombination processes. The RAD51/52-dependent DNA repair pathway is highly regulated and includes many proteins, some of which may be potential substrates for BAP1-mediated ubiquitin hydrolysis. RAD23, which associates with the RAD51/52 complex contains an amino-terminal ubiquitin-like domain which is required for RAD23 function and double-strand break repair (Watkins et al., 1993). Recently, a human ubiquitin-like protein, UBL-1, was isolated as a protein which binds directly to the human RAD51/RAD52 complex (Shen et al., 1996). Interestingly, the yeast homologue of UBL1 is SMT3, which functionally associates with the yeast centromere protein MIF2, a protein required for proper chromosome segregation (Brown, 1995; Brown et al., 1993). It is possible that BAP1, which is co-expressed with BRCA1 in testis, may regulate the recombination/repair functions of the BRCA1/RAD52 complex by targeting either RAD23 or UBL1 for ubiquitin hydrolysis.

Conclusions

We have identified a novel protein, BAP1, which binds to the BRCA1 RING finger domain (BRCA1-RF). BAP1 is a new, nuclear-localized, ubiquitin carboxyl-terminal hydrolase suggesting that deubiquitinating enzymes may play an important role in BRCA1 function. BAP1 binds to the wild-type BRCA1-RF both *in vitro* and *in vivo*, but not to mutant BRCA1-RF's found in tumors from breast cancer kindreds, or to related RING-finger proteins. BAP1 enhances BRCA1-mediated tumor suppression in colony formation assays and this activity requires the enzymatic function. BAP1 was mapped to human chromosome 3p21; rearrangements and intragenic mutations of BAP1 have been found in lung carcinomas, including a homozygous deletion. BAP1 is the first nuclear-localized ubiquitin carboxy-terminal hydrolase (of the UCH class) to be identified and may be a new tumor suppressor gene which functions in the BRCA1 growth control pathway.

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Appendices

Figures

Figure 1 Structural features of the BRCA1 gene product. A) Alignment of the RING finger domains of human and mouse BRCA1 (AA's 21-67), RPT-1 (AA's 12-61; the most closely related RING finger) and BARD1 (AA's 47-89). Asterisks (*) identify the Zn-chelating amino acids that form the core of the RING finger. Boxed amino acids show regions of identity between the RING finger domains of human BRCA1 and the other proteins. Alignment performed by ClustalW (Thompson et al., 1994). B) The amino-terminal 100 amino acids of human BRCA1 (which includes the RING finger domain) or the indicated amino acids of the various BRCA1-RF mutants and controls were fused to the LexA DNA-binding domain. Expression of all fusions in yeast were confirmed by Western analysis. The LexA-BRCA1 fusion protein was used in the yeast 2-hybrid screen to identify interacting proteins.

Figure 2 Identification of the same BRCA1-RF-interacting protein from human and murine libraries that function in the yeast 2-hybrid system. A) The BRCA1-interacting protein specifically interacts with the BRCA1 RING finger domain. Two hybrid screens of a human B-cell library and a mouse embryo (9.5-10.5d) library identified a protein that interacted with wild-type BRCA1-RF, but not with BRCA1-del31 {a truncated BRCA1}, BRCA1(Cys64Gly) {a BRCA1-RF containing a point mutation}, RPT-1 {a RING finger closely resembling the BRCA1}, or RhoB {a non-related protein}. Dark color of yeast indicates transcription from the LacZ locus - a positive interaction. Clones obtained from the two libraries are described as partial BAP1 proteins with A.A.s in parentheses. h, human; m, mouse. B) BAP1 interaction requires its C-terminal domain. Murine clone mBAP1(596-721) defines the minimal interaction domain of BAP1. Mutants of this clone were generated by PCR-based deletion or point mutagenesis of mBAP1(596-721) as described in Materials and Methods. Each individual mutant was co-transformed with LexA-BRCA1-RF into L40 yeast and tested for interaction via its ability to activate transcription from the LacZ locus.

Figure 3 BAP1 is a novel ubiquitin carboxy-terminal hydrolase (UCH). A) The nucleotide and amino acid sequence of BAP1. The longest open reading frame which contained the amino acids defined by the human 2-hybrid fusion protein is 2188 nucleotides encoding 729 amino acids. The cDNA also contains 39 nucleotides of 5'UTR and 1705 nucleotides of 3'UTR. The enzymatic active site is contained within the first 250 amino acids; the active site residues are circled. The putative nuclear localization signals (NLS) are underlined, the highly acidic region is boxed with heavy lines, the interaction domain is boxed and the protein fragment used to generate BAP1 polyclonal antibodies is bracketed (A.A.'s 483-576). B and C) Comparison of BAP1 with other UCH's. UCH-CAEEL (genebank # Q09444), UCH DROME (genebank # P35122), UCHL-1 (genebank # P09936), UCHL-3 (genebank # P15374). The BLAST search algorithm was used to identify proteins closely related to BAP1 (Altschul et al., 1990). The UCH domain of four of these proteins were aligned with BAP1 using the CLUSTALW (ver.1.6) algorithm (Thompson et al., 1994). Areas of homology with other UCH's are boxed. Only CAEEL-CO8B11.7 showed any homology outside of the enzymatic region. The region necessary for the interaction with BRCA1 (AAs 598-729) is indicated in the diagram with light crosshatching.

Figure 4 Expression and enzymatic activity of the BAP1 Protein. A) BAP1 has an apparent molecular weight of 90 kDa. The BAP1 cDNA was transcribed and translated *in vitro* (BAP1 IVT) in the presence of ^{35}S-Methionine and immunoprecipitated with either pre-immune or anti-BAP1 seras. BAP1 was also immunoprecipitated from extracts of ^{35}S-labeled COS1 cells transfected with empty expression plasmid or with plasmid including the BAP1 cDNA. B) BAP1 has ubiquitin hydrolase activity. BAP1, or an enzymatically null mutant, BAP1(C91S), were expressed in bacteria by IPTG induction. Bacteria were harvested, lysed and supernatent and pellet fractions generated. Each fraction was then measured for UCH activity (bar diagram; n.d., not detected). Induction of protein was verified by SDS-PAGE of each fraction. Arrow indicates BAP1 and BAP1(C91S).

Figure 5 BAP1 and BRCA1 interact *in vitro*. A) The GST, GST-hBAP1(483-729) and GST-hBAP1(483-594) fusion proteins were expressed and purified as described in Materials and Methods. Each lane represents the protein from 20 μL of a 50:50 resin slurry separated by SDS-PAGE and visualized by Coomassie blue staining. B) BRCA1 and GST-hBAP1(483-729) interact *in vitro*. The Glutathione-Sepharose resins containing the proteins shown in Figure 5A were incubated in batch with *in vitro* -expressed, ^{35}S-labeled, BRCA1. After extensive washing, the proteins which remained bound were analyzed by SDS-PAGE and fluorography. Lane 1, Input, 2% of the labeled BRCA1 used in the associations with the glutathione resins. Lane 2, molecular weight markers. Lane 3, proteins bound to GST alone. Lane 4, proteins bound to GST-hBAP-1(483-729). Lane 5, proteins bound to GST-hBAP-1(483-594), a fusion protein lacking the BRCA1 interaction domain. Arrow indicates the BRCA1 protein. C) LexA-BRCA1-RF and GST-hBAP1(483-729) interact *in vitro*. Glutathione-Sepharose resins containing the GST or GST-hBAP1(483-729) proteins were incubated in batch with *in vitro* -expressed, ^{35}S-labeled, LexA-BRCA1-RF. After extensive washing, the proteins which remained bound were analyzed by SDS-PAGE and fluorography. Lane 1, proteins bound to GST alone. Lane 2, proteins bound to GST-hBAP-1(483-729). Lane 3, Input, 5% of the labeled LexA-BRCA1-RF used in the associations with the glutathione resins. Bracket indicates the LexA-BRCA1-RF protein.

Figure 6 Tissue expression and subcellular localization of BAP1. A) Multiple tissue northern blots were obtained from Clonetech (Palo Alto, CA) and contain RNA from the indicated tissues. The blots were probed with the hBAP-1(483-729) cDNA (nucleotides 1488 to 3525) as described by the manufacturer. Blots were also subsequently probed with a muscle actin cDNA. B) BAP1 is a nuclear protein. Detection of BAP1 by confocal microscopy in Hep2 cells transfected with the BAP1 cDNA. Cells were stained with αBAP1 antibody as described in Materials and Methods.

Figure 7 BAP1 enhances BRCA1-mediated growth suppression. A) MCF7 cells were co-transfected with each of the plasmid constructs shown. Cells were then harvested and $5X10^5$ cells were plated in duplicate into G418-containing medium. Twenty one to 28 days later, cells were stained and colonies counted. The experiment was repeated 4 times with similar results. B) Quantitation of the results from (A).

Figure 8 BAP1 maps to Chromosome 3p21.3. Fluorescent *In Situ* Hybridization (FISH) of partial metaphases using biotin-labeled BAP1 cDNA. A) the specific FISH signals on chromosome 3 (arrows), with B) the simultaneously BAPI-stained chromosomes, and C) a chromosome ideogram with the localization of BAP1 based on the DAPI-band pattern and FLpter value. The horizontal box indicates the variation in FLpter values on individual chromosomes.

Figure 9 Mutational analysis of lung carcinomas. A) Southern blot hybridization with BamH1 digestion showing four distinct bands at 7.5kb, 4.0kb, 3.0kb, and 2.4kb detected by a full-length BAP1 probe. The non-small cell lung cancer NCI-H226 line shows an absence of the 7.5kb, 4.0kb, and 3.0kb bands. An aberrant 2.6kb band is detected in the H226 cell line. B) Northern blot hybridization showing a 4kb message which is absent in H226 and non-small cell lung cancer NCI-H1466 line. A fainter (5.0 kb) band is visible corresponding to cross hybridization with the 28S ribosomal component. C) SSCP analysis showing a homozygous shift in H1466 detected by RT-PCR amplification with primer set F (see text for primer sequences). Automated sequencing revealed an 8 base pair frameshift deletion in the H1466 cDNA, predicted to encode a 393 amino acid protein.

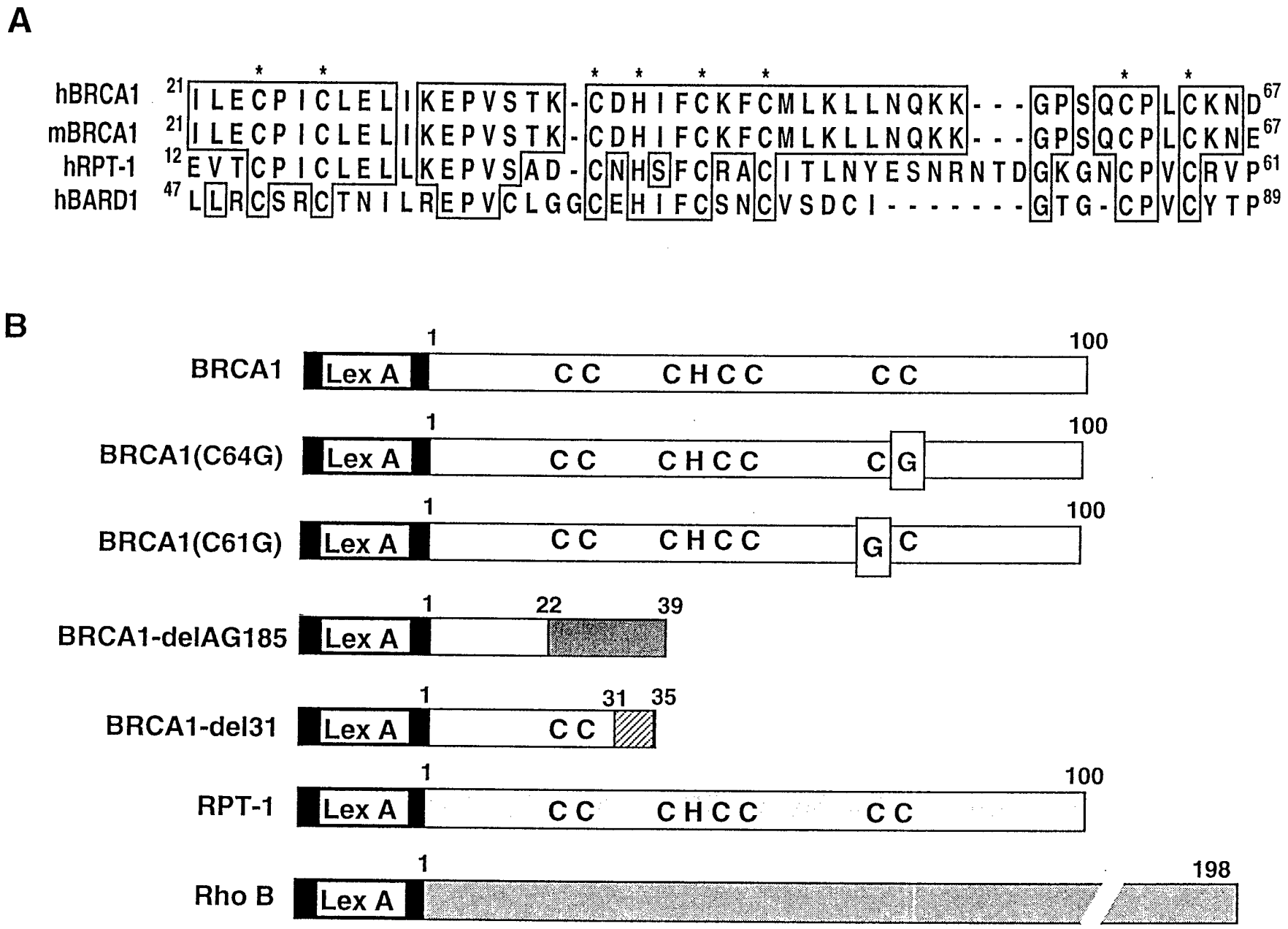
A
hBRCA1 21 I L E C P I C L E L I K E P V S T K - C D H I F C K F C M L K L L N Q K K - - - G P S Q C P L C K N D 67
mBRCA1 21 I L E C P I C L E L I K E P V S T K - C D H I F C K F C M L K L L N Q K K - - - G P S Q C P L C K N E 67
hRPT-1 12 E V T C P I C L E L L K E P V S A D - C N H S F C R A C I T L N Y E S N R N T D G K G N C P V C R V P 61
hBARD1 47 L L R C S R C T N I L R E P V C L G G C E H I F C S N C V S D C I - - - - - - - G T G - C P V C Y T P 89
B
BRCA1
Lex A
C C C H C C C C
1
100
BRCA1(C64G)
Lex A
C C C H C C C G
1
100
BRCA1(C61G)
Lex A
C C C H C C G C
1
100
BRCA1-delAG185
Lex A
1
22
39
BRCA1-del31
Lex A
C C
1
31
35
RPT-1
Lex A
C C C H C C C C
1
100
Rho B
Lex A
1
198

Figure 1

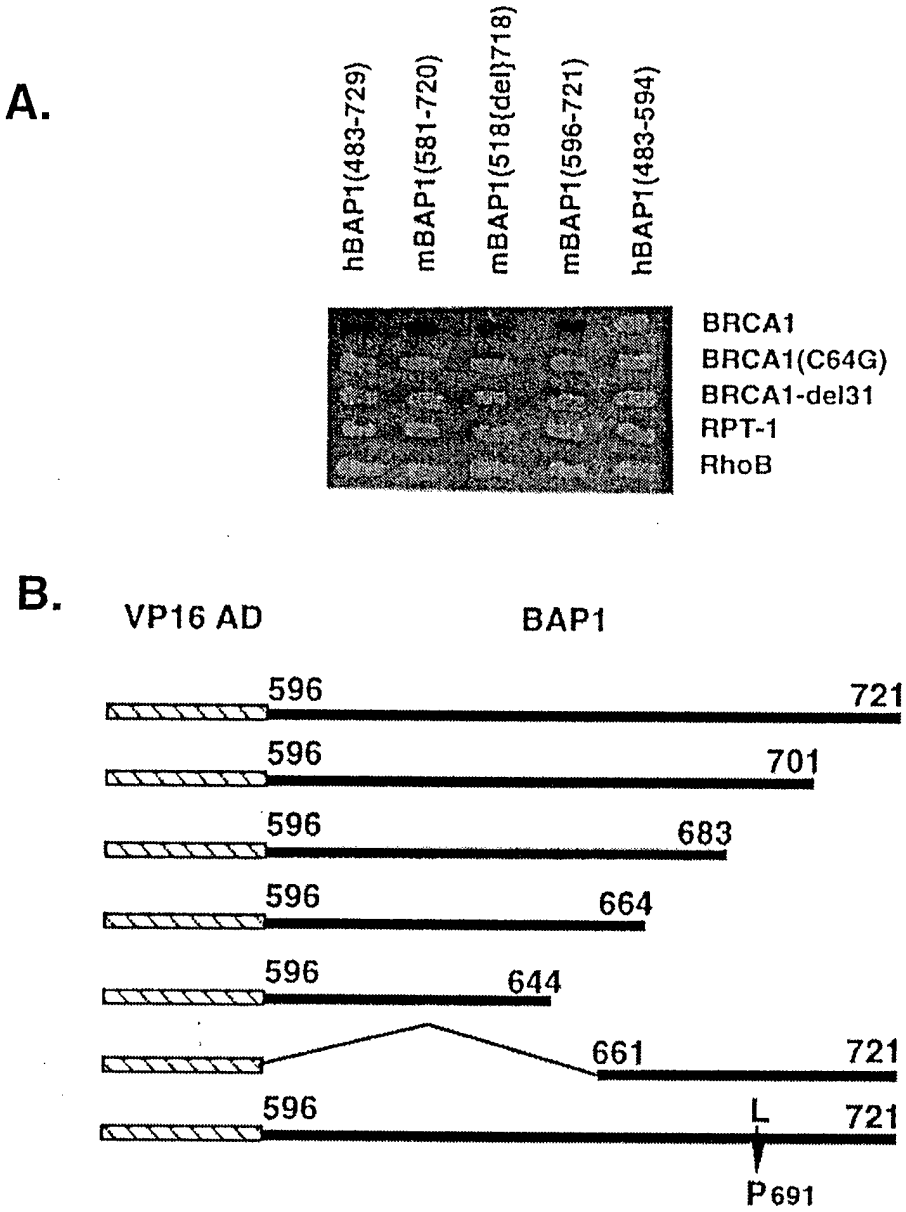
A.
hBAP1(483-729)
mBAP1(581-720)
mBAP1(518{del}718)
mBAP1(596-721)
hBAP1(483-594)
BRCA1
BRCA1(C64G)
BRCA1-del31
RPT-1
RhoB
B.
VP16 AD
BAP1
596
721
596
701
596
683
596
664
596
644
661
721
596
L
721
P691

Figure 2

A. BAP1

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1     GGCAC GAGGC ATGGC GCTGA GGGGC CGCCC CGCGG GAAG

40    ATG AAT AAG GGC TGG CTG GAG CTG GAG AGC GAC CCA GGC CTC TTC ACC
 1     M   N   K   G   W   L   E   L   E   S   D   P   G   L   F   T
88    CTG CTC GTG GAA GAT TTC GGT GTC AAG GGG GTG CAA GTG GAG GAG ATC
17    L   L   V   E   D   F   G   V   K   G   V   Q   V   E   E   I
136   TAC GAC CTT CAG AGC AAA TGT CAG GGC CCT GTA TAT GGA TTT ATC TTC
33    Y   D   L   Q   S   K   C   Q   G   P   V   Y   G   F   I   F
184   CTG TTC AAA TGG ATC GAA GAG CGC CGG TCC CGG CGA AAG GTC TCT ACC
49    L   F   K   W   I   E   E   R   R   S   R   R   K   V   S   T
232   TTG GTG GAT GAT ACG TCC GTG ATT GAT GAT GAT ATT GTG AAT AAC ATG
65    L   V   D   D   T   S   V   I   D   D   D   I   V   N   N   M
280   TTC TTT GCC CAC CAG CTG ATA CCC AAC TCT TGT GCA ACT CAT GCC TTG
81    F   F   A   H   Q   L   I   P   N   S   C   A   T   H   A   L
328   CTG AGC GTG CTC CTG AAC TGC AGC AGC GTG GAC CTG GGA CCC ACC CTG
97    L   S   V   L   L   N   C   S   S   V   D   L   G   P   T   L
376   AGT CGC ATG AAG GAC TTC ACC AAG GGT TTC AGC CCT GAG AGC AAA GGA
113   S   R   M   K   D   F   T   K   G   F   S   P   E   S   K   G
424   TAT GCG ATT GGC AAT GCC CCG GAG TTG GCC AAG GCC CAT AAT AGC CAT
129   Y   A   I   G   N   A   P   E   L   A   K   A   H   N   S   H
472   GCC AGG CCC GAG CCA CGC CAC CTC CCT GAG AAG CAG AAT GGC CTT AGT
145   A   R   P   E   P   R   H   L   P   E   K   Q   N   G   L   S
520   GCA GTG CGG ACC ATG GAG GCG TTC CAC TTT GTC AGC TAT GTG CCT ATC
161   A   V   R   T   M   E   A   F   H   F   V   S   Y   V   P   I
568   ACA GGC CGG CTC TTT GAG CTG GAT GGG CTG AAG GTC TAC CCC ATT GAC
177   T   G   R   L   F   E   L   D   G   L   K   V   Y   P   I   D
616   CAT GGG CCC TGG GGG GAG GAC GAG GAG TGG ACA GAC AAG GCC CGG CGG
193   H   G   P   W   G   E   D   E   E   W   T   D   K   A   R   R
664   GTC ATC ATG GAG CGT ATC GGC CTC GCC ACT GCA GGG GAG CCC TAC CAC
209   V   I   M   E   R   I   G   L   A   T   A   G   E   P   Y   H
712   GAC ATC CGC TTC AAC CTG ATG GCA GTG GTG CCC GAC CGC AGG ATC AAG
225   D   I   R   F   N   L   M   A   V   V   P   D   R   R   I   K
760   TAT GAG GCC AGG CTG CAT GTG CTG AAG GTG AAC CGT CAG ACA GTA CTA
241   Y   E   A   R   L   H   V   L   K   V   N   R   Q   T   V   L
808   GAG GCT CTG CAG CAG CTG ATA AGA GTA ACA CAG CCA GAG CTG ATT CAG
257   E   A   L   Q   Q   L   I   R   V   T   Q   P   E   L   I   Q
856   ACC CAC AAG TCT CAA GAG TCA CAG CTG CCT GAG GAG TCC AAG TCA GCC
273   T   H   K   S   Q   E   S   Q   L   P   E   E   S   K   S   A
904   AGC AAC AAG TCC CCG CTG GTG CTG GAA GCA AAC AGG GCC CCT GCA GCC
289   S   N   K   S   P   L   V   L   E   A   N   R   A   P   A   A
952   TCT GAG GGC AAC CAC ACA GAT GGT GCA GAG GAG GCG GCT GGT TCA TGC
305   S   E   G   N   H   T   D   G   A   E   E   A   A   G   S   C
1000  GCA CAA GCC CCA TCC CAC AGC CCT CCC AAC AAA CCC AAG CTA GTG GTG
321   A   Q   A   P   S   H   S   P   P   N   K   P   K   L   V   V
1048  AAG CCT CCA GGC AGC AGC CTC AAT GGG GTT CAC CCC AAC CCC ACT CCC
337   K   P   P   G   S   S   L   N   G   V   H   P   N   P   T   P
1096  ATT GTC CAG CGG CTG CCG GCC TTT CTA GAC AAT CAC AAT TAT GCC AAG
353   I   V   Q   R   L   P   A   F   L   D   N   H   N   Y   A   K
1144  TCC CCC ATG CAG GAG GAA GAA GAC CTG GCG GCA GGT GTG GGC CGC AGC
369   S   P   M   Q   E   E   E   D   L   A   A   G   V   G   R   S
1192  CGA GTT CCA GTC CGC CCA CCC CAG CAG TAC TCA GAT GAT GAG GAT GAC
385   R   V   P   V   R   P   P   Q   Q   Y   S   D   D   E   D   D
1240  TAT GAG GAT GAC GAG GAG GAT GAC GTG CAG AAC ACC AAC TCT GCC CTT
401   Y   E   D   D   E   E   D   D   V   Q   N   T   N   S   A   L
1288  AGG TAT AAG GGG AAG GGA ACA GGG AAG CCA GGG GCA TTG AGC GGT TCT
417   R   Y   K   G   K   G   T   G   K   P   G   A   L   S   G   S
1336  GCT GAT GGG CAA CTG TCA GTG CTG CAG CCC AAC ACC ATC AAC GTC TTG
433   A   D   G   Q   L   S   V   L   Q   P   N   T   I   N   V   L
1384  GCT GAG AAG CTC AAA GAG TCC CAG AAG GAC CTC TCA ATT CCT CTG TCC
449   A   E   K   L   K   E   S   Q   K   D   L   S   I   P   L   S
1432  ATC AAG ACT AGC AGC GGG GCT GGG AGT CCG GCT GTG GCA GTG CCC ACA
465   I   K   T   S   S   G   A   G   S   P   A   V   A   V   P   T
1480  CAC TCG CAG CCC TCA CCC ACC CCC AGC AAT GAG AGT ACA GAC ACG GCC
481   H   S   Q   P   S   P   T   P   S   N   E   S   T   D   T   A
1528  TCT GAG ATC GGC AGT GCT TTC AAC TCG CCA CTG CGC TCG CCT ATC CGC
497   S   E   I   G   S   A   F   N   S   P   L   R   S   P   I   R
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Figure 3A

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1576 TCA GCC AAC CCG ACG CGG CCC TCC AGC CCT GTC ACC TCC CAC ATC TCC
513   S   A   N   P   T   R   P   S   S   P   V   T   S   H   I   S
1624 AAG GTG CTT TTT GGA GAG GAT GAC AGC CTG CTG CGT GTT GAC TGC ATA
529   K   V   L   F   G   E   D   D   S   L   L   R   V   D   C   I
1672 CGC TAC AAC CGT GCT GTC CGT GAT CTG GGT CCT GTC ATC AGC ACA GGC
545   R   Y   N   R   A   V   R   D   L   G   P   V   I   S   T   G
1720 CTG CTG CAC CTG GCT GAG GAT GGG GTG CTG AGT CCC CTG GCG CTG ACA
561   L   L   H   L   A   E   D   G   V   L   S   P   L   A   L   T
1768 GAG GGT GGG AAG GGT TCC TCG CCC TCC ATC AGA CCA ATC CAA GGC AGC
577   E   G   G   K   G   S   S   P   S   I   R   P   I   Q   G   S
1816 CAG GGG TCC AGC AGC CCA GTG GAG AAG GAG GTC GTG GAA GCC ACG GAC
593   Q   G   S   S   S   P   V   E   K   E   V   V   E   A   T   D
1864 AGC AGA GAG AAG ACG GGG ATG GTG AGG CCT GGC GAG CCC TTG AGT GGG
609   S   R   E   K   T   G   M   V   R   P   G   E   P   L   S   G
1912 GAG AAA TAC TCA CCC AAG GAG CTG CTG GCA CTG CTG AAG TGT GTG GAG
625   E   K   Y   S   P   K   E   L   L   A   L   L   K   C   V   E
1960 GCT GAG ATT GCA AAC TAT GAG GCG TGC CTC AAG GAG GAG GTA GAG AAG
641   A   E   I   A   N   Y   E   A   C   L   K   E   E   V   E   K
2008 AGG AAG AAG TTC AAG ATT GAT GAC CAG AGA AGG ACC CAC AAC TAC GAT
657   R   K   K   F   K   I   D   D   Q   R   R   T   H   N   Y   D
2056 GAG TTC ATC TGC ACC TTT ATC TCC ATG CTG GCT CAG GAA GGC ATG CTG
673   E   F   I   C   T   F   I   S   M   L   A   Q   E   G   M   L
2104 GCC AAC CTA GTG GAG CAG AAC ATC TCC GTG CGG CGG CGC CAA GGG GTC
689   A   N   L   V   E   Q   N   I   S   V   R   R   R   Q   G   V
2152 AGC ATC GGC CGG CTC CAC AAG CAG CGG AAG CCT GAC CGG CGG AAA CGC
705   S   I   G   R   L   H   K   Q   R   K   P   D   R   R   K   R
2200 TCT CGC CCC TAC AAG GCC AAG CGC CAG TGA
721   S   R   P   Y   K   A   K   R   Q   *

2230 GGACT GCTGG CCCTG ACTCT GCAGC CCACT CTTGC CGTGT GGCCC TCACC AGGGT
2285 CCTTC CCTGC CCCAC TTCCC CTTTT CCCAG TATTA CTGAA TAGTC CCAGC TGGAG
2340 AGTCC AGGCC CTGGG AATGG GAGGA ACCAG GCCAC ATTCC TTCCA TCGTG CCCTG
2395 AGGCC TGACA CGGCA GATCA GCCCC ATAGT GCTCA GGAGG CAGCA TCTGG AGTTG
2450 GGGCA CAGCG AGGTA CTGCA GCTTC CTCCA CAGCC GGCTG TGGAG CAGCA GGACC
2505 TGGCC CTTCT GCCTG GGCAG CAGAA TATAT ATTTT ACCTA TCAGA GACAT CTATT
2560 TTTCT GGGCT CCAAC CCAAC ATGCC ACCAT GTTGA CATAA GTTCC TACCT GACTA
2615 TGCTT TCTCT CCTAG GAGCT GTCCT GGTGG GCCCA GGTCC TTGTA TCATG CCACG
2670 GTCCC AACTA CAGGG TCCTA GCTGG GGGCC TGGGT GGGCC CTGGG CTCTG GGCCC
2725 TGCTG CTCTA GCCCC AGCCA CCAGC CTGTC CCTGT TGTAA GGAAG CCAGG TCTTC
2780 TCTCT TCATT CCTCT TAGGA GAGTG CCAAA CTCAG GGACC CAGCA CTGGG CTGGG
2835 TTGGG AGTAG GGTGT CCCAG TGGGG TTGGG GTGAG CAGGC TGCTG GGATC CCATG
2890 GCCTG AGCAG AGCAT GTGGG AACTG TTCAG TGGCC TGTGA ACTGT CTTCC TTGTT
2945 CTAGC CAGGC TGTTC AAGAC TGCTC TCCAT AGCAA GGTTC TAGGG CTCTT CGCCT
3000 TCAGT GTTGT GGCCC TAGCT ATGGG CCTAA ATTGG GCTCT AGGTC TCTGT CCCTG
3055 GCGCT TGAGG CTCAG AAGAG CCTCT GTCCA GCCCC TCAGT ATTAC CATGT CTCCC
3110 TCTCA GGGGT AGCAG AGACA GGGTT GCTTA TAGGA AGCTG GCACC ACTCA GCTCT
3165 TCCTG CTACT CCAGT TTCCT CAGCC TCTGC AAGGC ACTCA GGGTG GGGGA CAGCA
3220 GGATC AAGAC AACCC GTTGG AGCCC CTGTG TTCCA GAGGA CCTGA TGCCA AGGGG
3275 TAATG GGCCC AGCAG TGCCT CTGGA GCCCA GGCCC CAACA CAGCC CCATG GCCTC
3330 TGCCA GATGG CTTTG AAAAA GGTGA TCCAA GCAGG CCCCT TTATC TGTAC ATAGT
3385 GACTG AGTGG GGGGT GCTGG CAAGT GTGGC AGCTG CCTCT GGGCT GAGCA CAGCT
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3495 GAATC TCCTG AGAAA AAAAA AAAAA AAAAG G
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B.

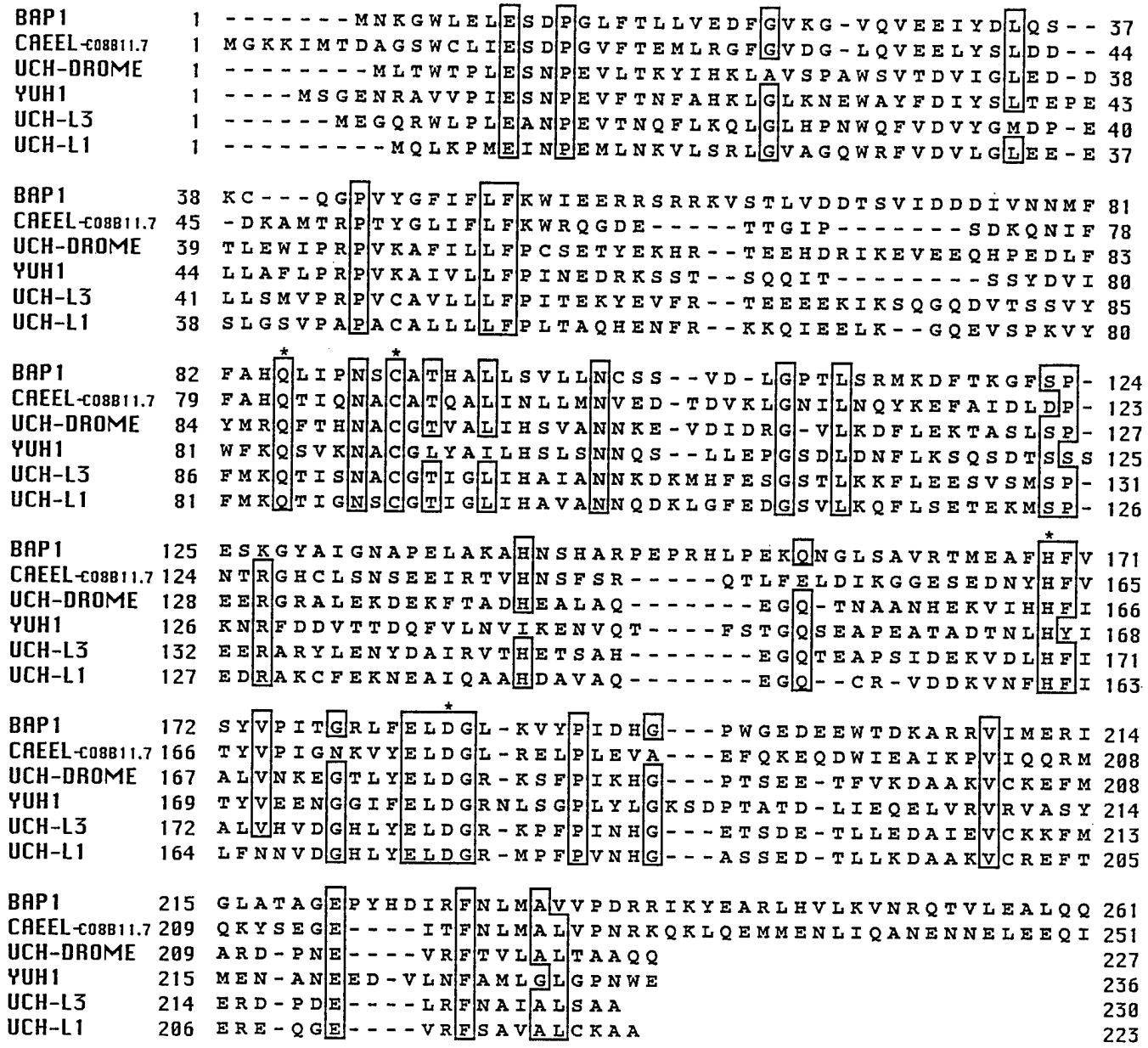

BAP1 1 -------MNKGWLELESDPGLFTLLVEDFGVKG-VQVEEIYDLQS-- 37
CAEEL-C08B11.7 1 MGKKIMTDAGSWCLIESDPGVFTEMLRGFGVDG-LQVEELYSLDD-- 44
UCH-DROME 1 ---------MLTWTPLESNPEVLTKYIHKLAVSPAWSVTDVIGLED-D 38
YUH1 1 ----MSGENRAVVPIESNPEVFTNFAHKLGLKNEWAYFDIYSLTEPE 43
UCH-L3 1 -------MEGQRWLPLEANPEVTNQFLKQLGLHPNWQFVDVYGMDP-E 40
UCH-L1 1 ----------MQLKPMEINPEMLNKVLSRLGVAGQWRFVDVLGLEE-E 37
BAP1 38 KC---QGPVYGFIFLFKWIEERRSRRKVSTLVDDTSVIDDDIVNNMF 81
CAEEL-C08B11.7 45 -DKAMTRPTYGLIFLFKWRQGDE-----TTGIP-------SDKQNIF 78
UCH-DROME 39 TLEWIPRPVKAFILLFPCSETYEKHR--TEEHDRIKEVEEQHPEDLF 83
YUH1 44 LLAFLPRPVKAIVLLFPINEDRKSST--SQQIT---------SSYDVI 80
UCH-L3 41 LLSMVPRPVCAVLLLFPITEKYEVFR--TEEEEKIKSQGQDVTSSVY 85
UCH-L1 38 SLGSVPAPACALLLLFPLTAQHENFR--KKQIEELK--GQEVSPKVY 80
BAP1 82 FAHQLIPNSCATHALLSVLLNCSS--VD-LGPTLSRMKDFTKGFSP- 124
CAEEL-C08B11.7 79 FAHQTIQNACATQALINLLMNVED-TDVKLGNILNQYKEFAIDLDP- 123
UCH-DROME 84 YMRQFTHNACGTVALIHSVANNKE-VDIDRG-VLKDFLEKTASLSP- 127
YUH1 81 WFKQSVKNACGLYAILHSLSNNQS--LLEPGSDLDNFLKSQSDTSSS 125
UCH-L3 86 FMKQTISNACGTIGLIHAIANNKDKMHFESGSTLKKFLEESVSMSP- 131
UCH-L1 81 FMKQTIGNSCGTIGLIHAVANNQDKLGFEDGSVLKQFLSETEKMSP- 126
BAP1 125 ESKGYAIGNAPELAKAHNSHARPEPRHLPEKQNGLSAVRTMEAFHFV 171
CAEEL-C08B11.7 124 NTRGHCLSNSEEIRTVHNSFSR-----QTLFELDIKGGESEDNYHFV 165
UCH-DROME 128 EERGRALEKDEKFTADHEALAQ--------EGQ-TNAANHEKVIHHFI 166
YUH1 126 KNRFDDVTTDQFVLNVIKENVQT----FSTGQSEAPEATADTNLHYI 168
UCH-L3 132 EERARYLENYDAIRVTHETSAH--------EGQTEAPSIDEKVDLHFI 171
UCH-L1 127 EDRAKCFEKNEAIQAAHDAVAQ--------EGQ--CR-VDDKVNFHFI 163
BAP1 172 SYVPITGRLFELDGL-KVYPIDHG---PWGEDEEWTDKARRVIMERI 214
CAEEL-C08B11.7 166 TYVPIGNKVYELDGL-RELPLEVA---EFQKEQDWIEAIKPVIQQRM 208
UCH-DROME 167 ALVNKEGTLYELDGR-KSFPIKHG---PTSEE-TFVKDAAKVCKEFM 208
YUH1 169 TYVEENGGIFELDGRNLSGPLYLGKSDPTATD-LIEQELVRVRVASY 214
UCH-L3 172 ALVHVDGHLYELDGR-KPFPINHG---ETSDE-TLLEDAIEVCKKFM 213
UCH-L1 164 LFNNVDGHLYELDGR-MPFPVNHG---ASSED-TLLKDAAKVCREFT 205
BAP1 215 GLATAGEPYHDIRFNLMAVVPDRRIKYEARLHVLKVNRQTVLEALQQ 261
CAEEL-C08B11.7 209 QKYSEGE-----ITFNLMALVPNRKQKLQEMMENLIQANENNELEEQI 251
UCH-DROME 209 ARD-PNE-----VRFTVLALTAAQQ 227
YUH1 215 MEN-ANEED-VLNFAMLGLGPNWE 236
UCH-L3 214 ERD-PDE-----LRFNAIALSAA 230
UCH-L1 206 ERE-QGE-----VRFSAVALCKAA 223
BAP1 630 KELLALLKCVEAEIANYEACLKEEVEKRKKFKID 633
CAEEL-C08B11.7 238 LIQANENNELEEQIADLNKAIADEDYKMEMYRKE 271
BAP1 634 DQRRTHNYDEFICTFISMLAQEGMLANLVEQNIS 667
CAEEL-C08B11.7 272 NNRRRHNYTPFVIELMKILAKEGKLVGLVDNAYQ 305
BAP1 668 VRRRQGVSIGRLHKQRKPDRRKRSRPYKAKRQ 729
CAEEL-C08B11.7 306 AAK-EK-S--KLNTDITKLELKRKQ 326

Figure 3B

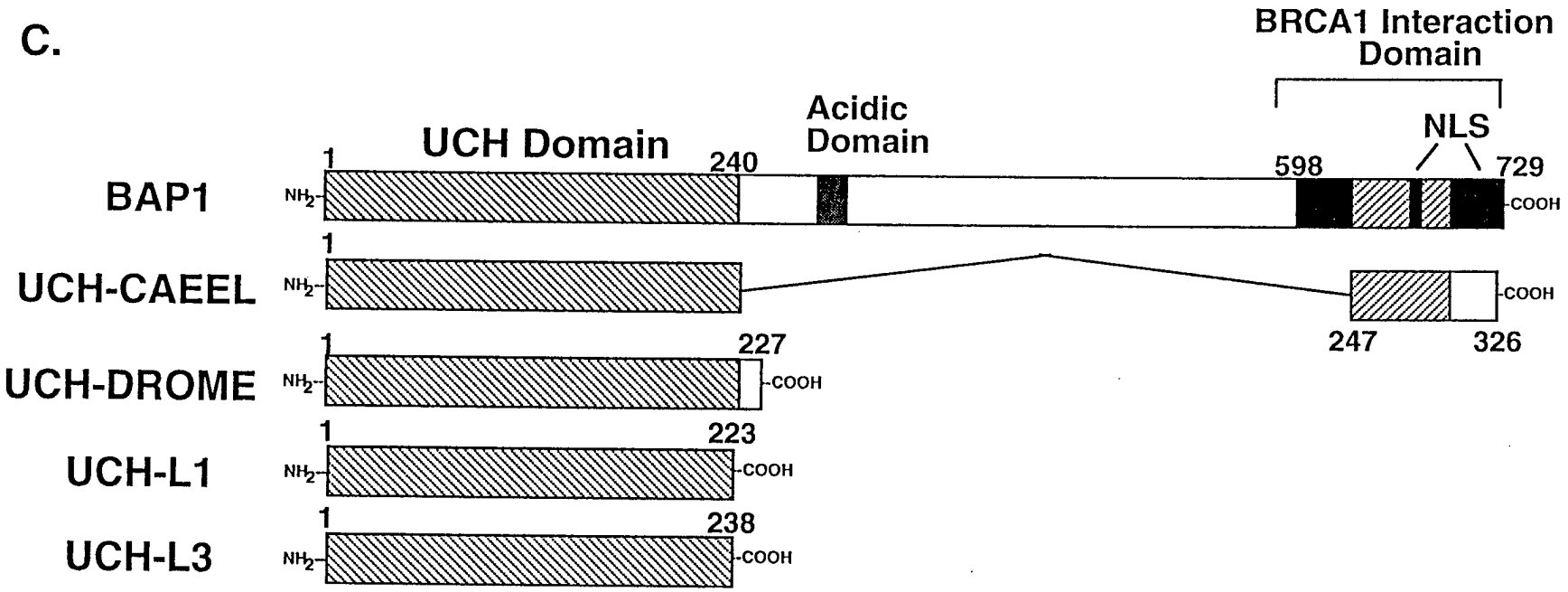

C.
BRCA1 Interaction Domain
UCH Domain
Acidic Domain
NLS
BAP1
UCH-CAEEL
UCH-DROME
UCH-L1
UCH-L3
1
240
598
729
227
223
238
247
326
NH2
COOH

Figure 3C

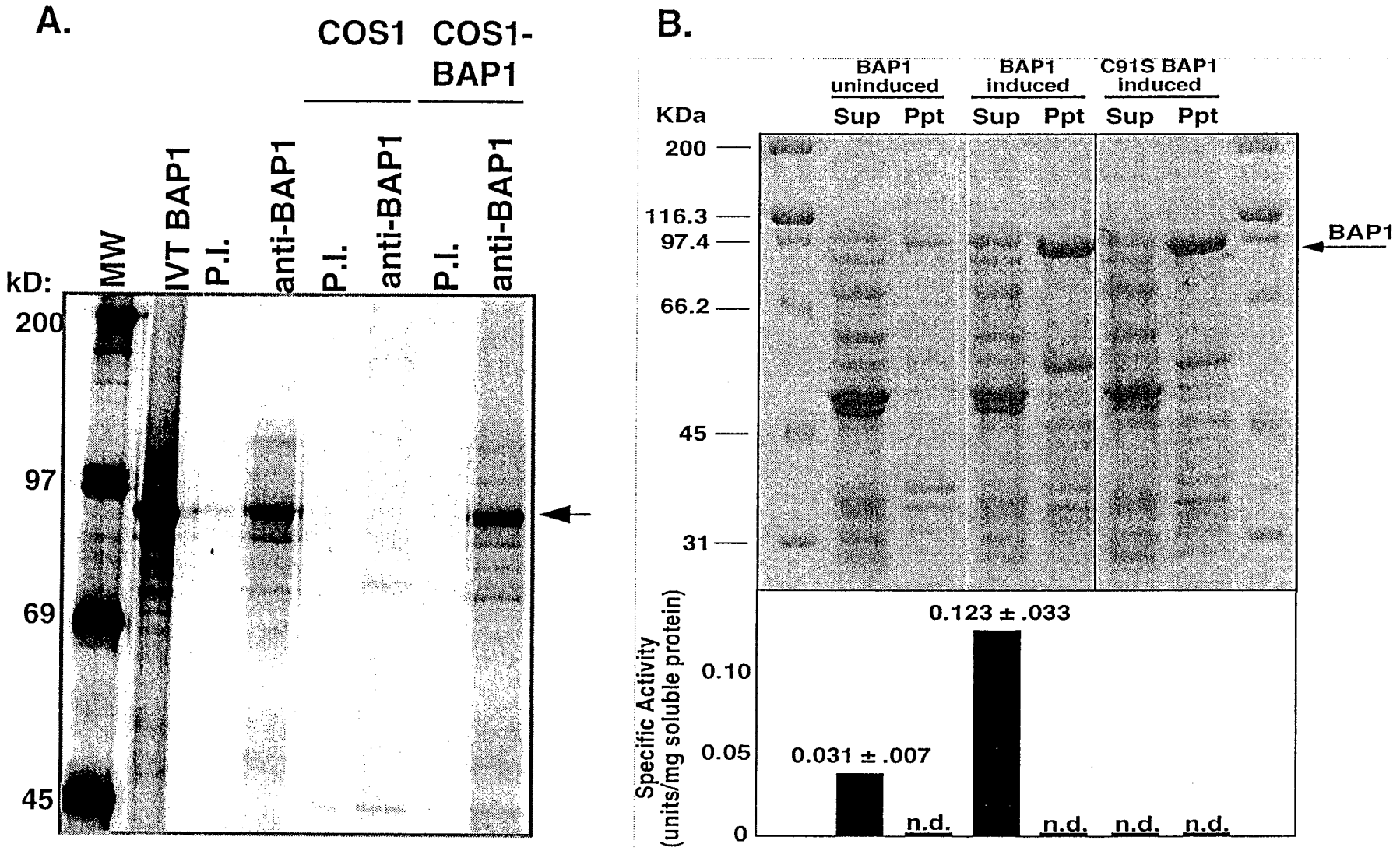

A.
COS1
COS1-BAP1
kD:
MW
IVT BAP1
P.I.
anti-BAP1
P.I.
anti-BAP1
P.I.
anti-BAP1
200
97
69
45
B.
BAP1 uninduced
BAP1 induced
C91S BAP1 induced
KDa
Sup
Ppt
Sup
Ppt
Sup
Ppt
200
116.3
97.4
66.2
45
31
BAP1
Specific Activity (units/mg soluble protein)
0.123 ± .033
0.10
0.05
0.031 ± .007
0
n.d.
n.d.
n.d.
n.d.

Figure 4

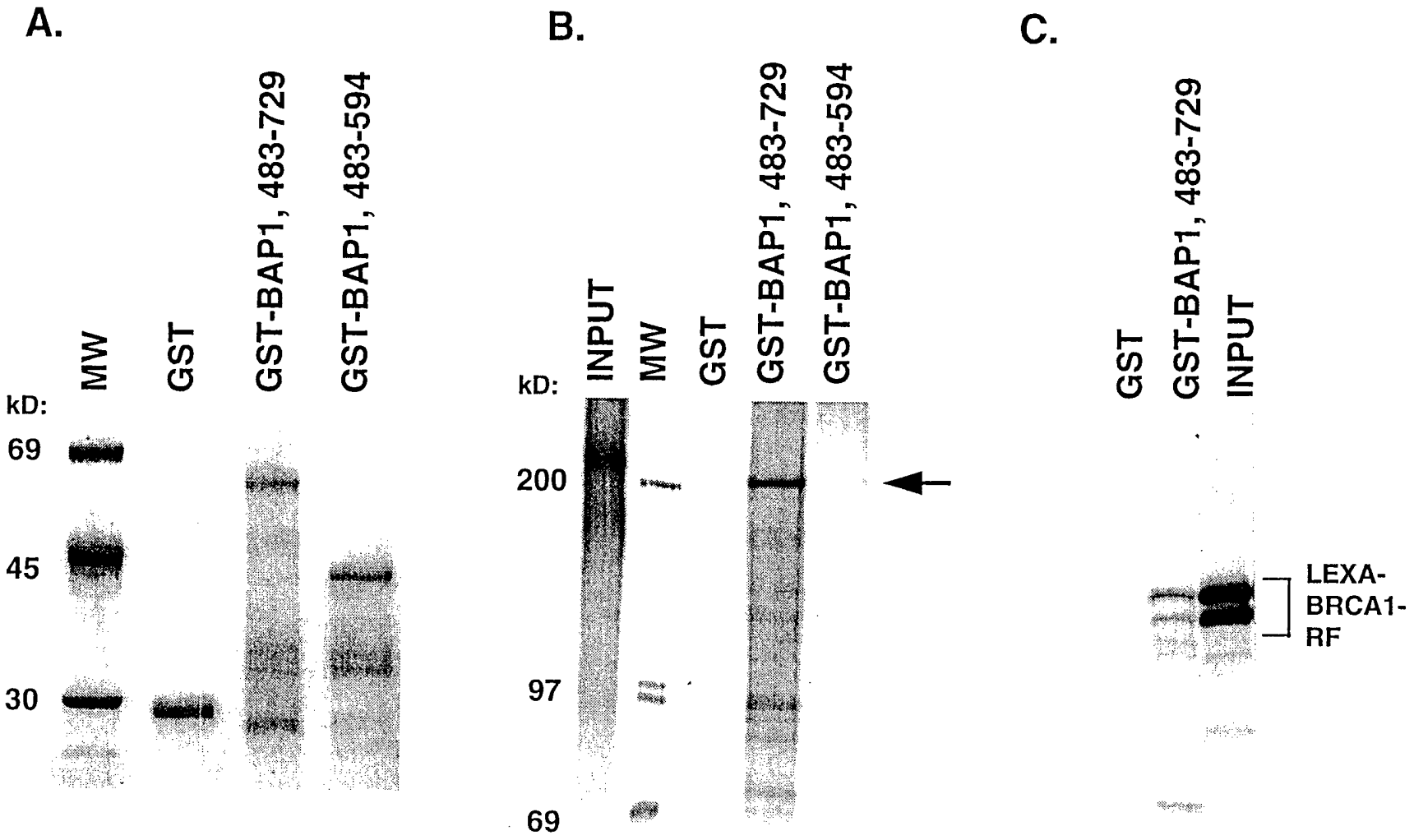

A.
MW
GST
GST-BAP1, 483-729
GST-BAP1, 483-594
kD:
69
45
30
B.
INPUT
MW
GST
GST-BAP1, 483-729
GST-BAP1, 483-594
kD:
200
97
69
C.
GST
GST-BAP1, 483-729
INPUT
LEXA-
BRCA1-
RF

Figure 5

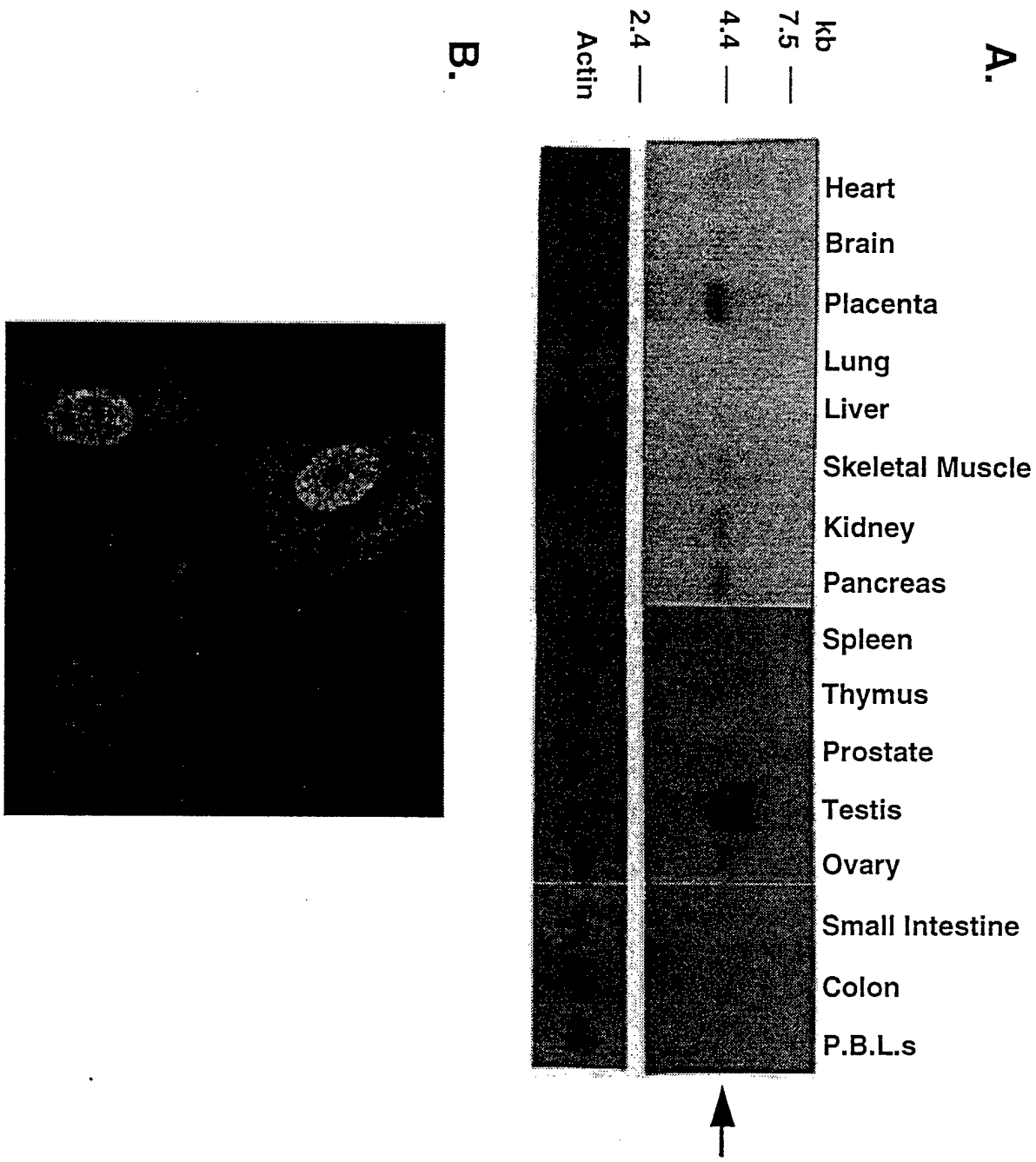

A.
kb
7.5
4.4
2.4
Actin
B.
Heart
Brain
Placenta
Lung
Liver
Skeletal Muscle
Kidney
Pancreas
Spleen
Thymus
Prostate
Testis
Ovary
Small Intestine
Colon
P.B.L.s

Figure 6

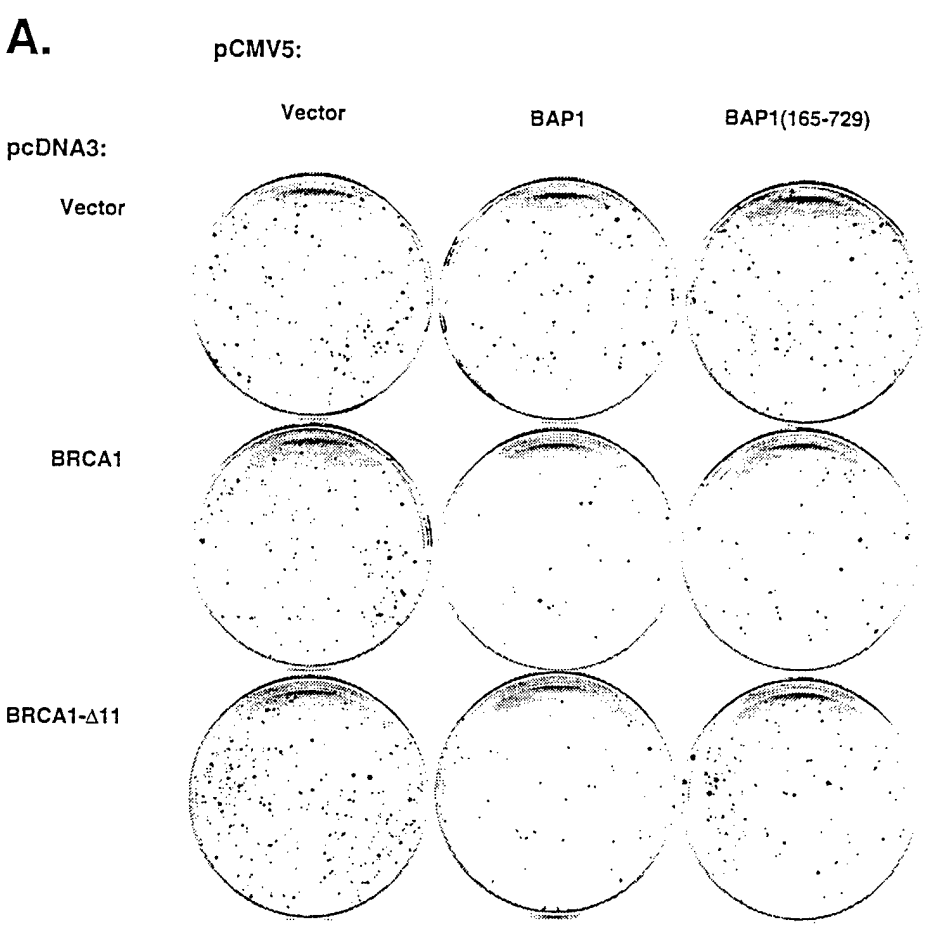

A.
pCMV5:
Vector
BAP1
BAP1(165-729)
pcDNA3:
Vector
BRCA1
BRCA1-Δ11
B.
Percent of Vector
Colony Number
pcDNA3
pCMV5
0
20
40
60
80
100
120
—
—
BRCA1
—
BRCA1
BAP1
BRCA1
BAP1(165-729)
—
—
BRCA1-Δ11
—
BRCA1-Δ11
BAP1
BRCA1-Δ11
BAP1(165-729)
—
—
—
BAP1
—
BAP1(165-729)

Figure 7

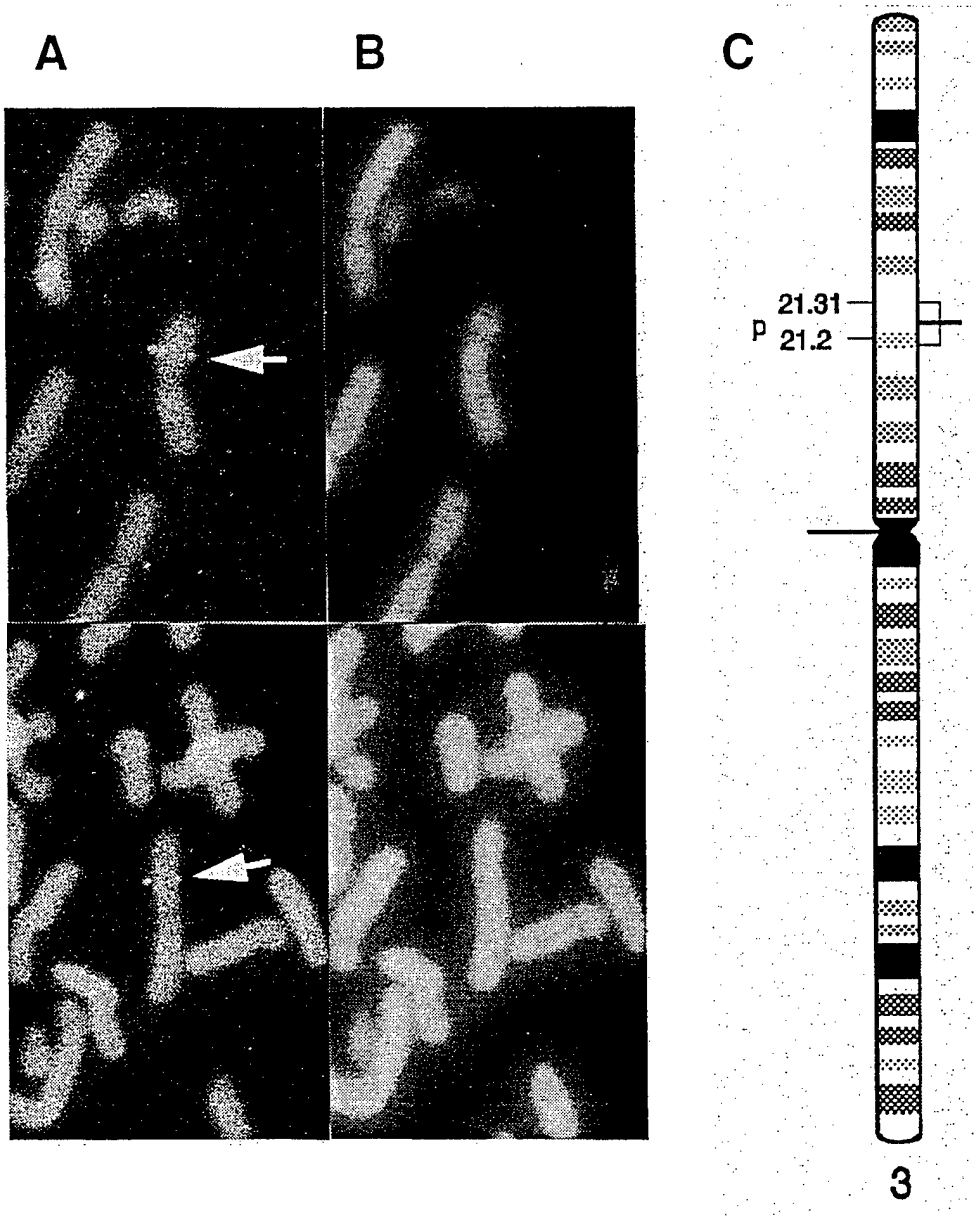
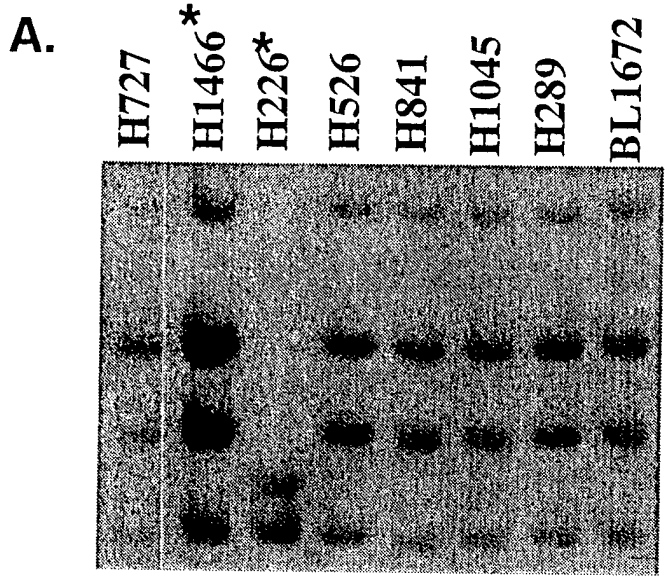
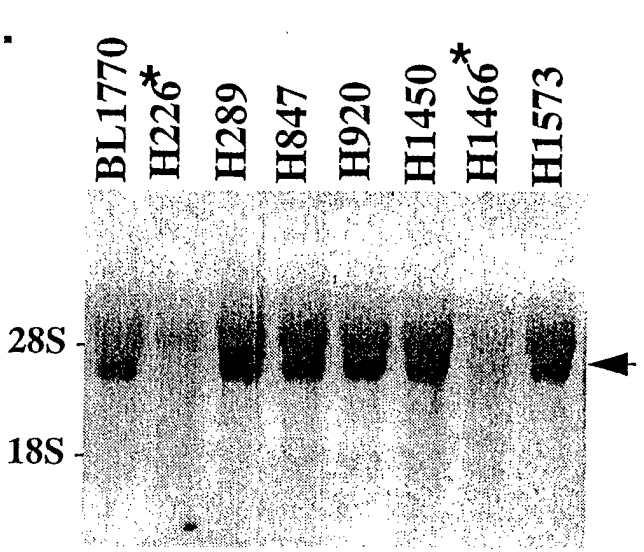
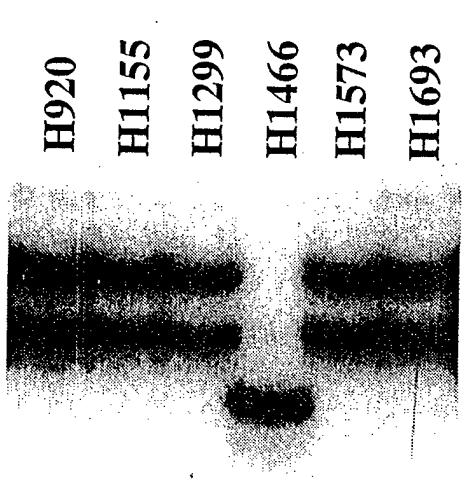


Figure 8







Normal:

5'...GGC CGC AGC CGA GTT CCA GTC..3'

... *G R S R V P V* 729aa

H1466: 1198-1205 del CGAGTTCC

5'...GGC CGC AGC-------(del)------AGT..3'

... *G R S S* 393aa

Figure 9